Glucocorticoids And Angiogenesis

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Doctor of Philosophy

University of Edinburgh

2005

Abstract

Angiogenesis is the creation of new blood vessels from the existing vasculature that occurs through a complex series of interactions and is tightly regulated. Glucocorticoids are acknowledged to inhibit angiogenesis at high concentrations but whether endogenous glucocorticoid concentrations are sufficient to regulate angiogenesis is unclear. Adrenal synthesis is not the sole determinant of glucocorticoid tissue concentration however and 11βhydroxysteroid dehydrogenase type 1 (11βHSD1) that is present in the vessel wall may regenerate active glucocorticoids from 11keto-substrates and therefore amplify local tissue concentrations. Thus it was hypothesised that endogenous glucocorticoids regulated by 11βHSD1 inhibit angiogenesis.

In vitro C57Bl6 mouse aortic ring cultures established that physiologically relevant concentrations of glucocorticoids inhibit angiogenesis in a glucocorticoid receptor dependent manner. In addition vascular 11βHSD1 was found to modulate glucocorticoid-induced angiostasis, for 11dehydrocorticosterone although angiostatic in C57Bl6 aortae did not inhibit angiogenesis in11βHSD1 deficient animals.

In vivo using polyester sponge subcutaneous implants in mice, endogenous glucocorticoids were found to inhibit angiogenesis: sponges in adrenalectomised C57Bl6 mice grew more vessels compared to sponges from sham-operated animals. 11βHSD1 regulated the angiostatic effects of glucocorticoids, for cortisone (the human equivalent of 11dehydrocorticosterone), although angiostatic in wild types did not inhibit angiogenesis in 11βHSD1 deficient mice. *In vivo* it was also noted

that in placebo-impregnated sponges angiogenesis was greater in 11βHSD1 deficient compared to C57Bl6 mice.

In pathology in cutaneous wounds and infarcted myocardium, endogenous glucocorticoids were found to inhibit angiogenesis. RU38486 (a glucocorticoid receptor antagonist) enhanced angiogenesis in both tissues in comparison to placebo treatment. In similar studies in C57Bl6 or 11βHSD1 deficient mice, 11βHSD1 was found to tonically repress angiogenesis and impair left ventricular remodelling post infarction. Thus 11βHSD1 deficient mice had increased myocardial revascularisation and preserved left ventricular function.

In conclusion, by using *in vitro, in vivo* and *pathological* models, endogenous glucocorticoids were seen to inhibit angiogenesis. In addition 11βHSD1 regeneration of glucocorticoids tonically repressed angiogenesis and influenced left ventricular remodelling post myocardial infarction. Thus 11βHSD1 appears to be an attractive therapeutic target for the management of tissue revascularisation.

Declaration

I declare that I have written this thesis and that the data presented represent my own work with the exceptions listed below:

Dr A Dover and Mrs Eileen Miller of the Centre for Cardiovascular Sciences University of Edinburgh performed the aortic ring activity assay experiments.

Miss Katherine Shaw cultured human umbilical vein endothelial cells and made them available for a series of experiments to measure intracellular calcium responses to VEGF.

Dr Patrick Hadoke performed adrenalectomy procedures.

I declare that this work has not been submitted for any other degree.

Gary Robert Small

Acknowledgements

I am most grateful for the tuition, advice and encouragement that have enabled this thesis to be studied. In particular I would like to pay tribute to the help of my family, my supervisors and the centre of cardiovascular sciences.

This work could not have been performed without the support from my wife Jenny and my children, thank you for your prayers and the endless supply of cups of tea during thesis writing.

Professor Brain Walker and Dr Patrick Hadoke have supervised, corrected and instructed my raw ideas and have been instrumental in encouraging my interest in clinical science. They have formed an exellent team I thank them for their many lessons, I look forward to potential collaborations and wish them every suceess in the future.

My thesis was undertaken at a time of change in the centre of cardiovascular sciences in Edinburgh and much of the work was inter-departmental. I am therefore most grateful for the cooperation and advice I have received from Dr Janice Patterson and Dr Gillian Gray from Biomedical Sciences, Dr Paul Perry MRC Human Genetics and staff at the Biomedical Research Facility. I should like to thank Professor Jonathan Seckl for his tuition and support. In addition I have worked with many members of the endocrinology group and their instruction and assistance was gratefully appreciated.

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List of Abbreviations

A 11dehydrocorticosterone

ACTH Adrenocorticotrophic hormone

Ang Angiopoeitin

ANP Atrial Naturetic peptide

ANOVA Analysis of variance

ATP Adenosine inositol triphosphate

AVP Arginine vasopressin

B Corticosterone

BAECs Bovine aortic endothelial cells

BH_4 Tetrahydrobiopterin

CAM Chick allantoic membrane

CBG Corticosteroid-binding globulin

CNS Central nervous system

CRH Corticotrophin releasing hormone

DiI Ac LDL Fluoresent labeled acetylated low density lipoprotein

DMSO Dimethyl Sulphur oxide

DNA Deoxyribo-nucleic Acid

E Cortisone

EDTA Ethylene diamine tetra-acetic acid

EF Ejection fraction

ER Endoplasmic reticulum

ESA End systolic area

F Cortisol

FBS fetal bovine serum

FGF Fibroblast growth factor

FS Fractional shortening

hEGF Human recombinant epidermal growth factor

IGF-1 Human recombinant insulin like growth factor-1

GCs Glucocorticoids

GH Growth hormone

GR Glucocorticoid receptor

GRE Glucocorticoid response element

GTP Guanosine triphosphate

HIFs Hypoxia inducible factors

HPA axis Hypothalamic pituitary adrenal axis

HPLC High performance liquid chromatography

HUVECs Human umbilical vein endothelial cells

3α-HSD 3α–Hydroxysteroid dehydrogenase

11βHSD1 11beta Hydroxysteroid dehydrogenase type 1

11βHSD2 11beta Hydroxysteroid dehydrogenase type 2

6βOHASE 6β hydroxylase

20HSD 20α Hydroxysteroid dehydrogenase

IL1 Interleukin 1

IL-6 Interleukin 6

KRB Kreb's ringer bicarbonate

LC/MS/MS Liquid chromatography mass spectrometry

LH Luteinising hormone

LMDA Left main descending artery

L-NNA NωNitro-L-Arginine

LVEDD Left ventricular end diastolic dimensions

LVESD Left ventricular end systolic dimensions

MCID Micro computer imaging device

MCP-1 Monocyte chemotactic protein-1

MMP Matrix metalloproteinase

MR Mineralocorticoid receptor

mRNA Messenger RNA

NAD Nicotinamide adenine dinucleotide

NADPH Nicotinamide adenine dinucleotide phosphate

NFI Nuclear factor I

NF-κβ Nuclear factor κβ

NO Nitric Oxide

PA Plasminogen system

PAI-1 Plasminogen activator inhibitor

PBS Phosphate buffered saline

PDGF Platelet derived growth factor

PECAM-1 Platelet endothelial cell adhesion molecule-1

PEPCK Phosphoenolpyruvate carboxykinase

PGE_2 Prostaglandin E_2

POMC Pro-opiomelanocortin

PPAR Persoxisome proliferators-activated receptor

PWD Posterior wall thickness in end diastole

PWS Posterior wall thickness in end systole

RNA Ribo- nucleic acid

RT-PCR Reverse transcriptase polymerase chain reaction

SAME Syndrome of apparent mineralocorticoid excess

SCC Side chain cleavage

S1P1 spinogosine 1 phosphate 1

SEM Standard error of the mean

TBS Tris-buffered saline

TGFβ Tissue growth factor β

TIMPs Tissue inhibitors of matrix metalloproteinases

TNFα Tissue necrosis factor α

TNS Trypsin neutralizing solution

t-PA tissue plasminogen activator

TxA_2 Thromboxane A_2

VEGF Vascular endothelial growth factor

VSMC Vascular smooth muscle cell

VEGFR VEGF receptor

vWF von-Willebrand factor

u-PA urokinase plasminogen activator

List of Publications

Abstracts

Small GR, Hadoke PWF, Walker BR (2003) Local regeneration of glucocorticoids by 11βHSD1 in the blood vessel wall potentiates inhibition of new vessel formation in mouse aorta. Poster presented at Medical Research Society 2003

Small GR, Hadoke PWF, Walker BR (2003) Glucocorticoids inhibit angiogenesis in mouse aorta: a novel role for 11βHSD1 in modifying vascular structure. Poster presentation to the American Endocrine Society 85th meeting Philadelphia 2003

Small GR, Dover AR, Hadoke PWF, Walker BR (2004) Local regeneration of glucocorticoids by 11βHSD-1 within the vessel wall modulates angiogenesis. Oral presentation to the Joint meeting of Scottish Cardiovascular forum and Scottish Society of Experimental Medicine 2004

Small GR, Dover AR, Hadoke PWF, Walker BR (2004) Local regeneration of glucocorticoids by 11βHSD-1 within the vessel wall modulates angiogenesis in vitro and in vivo in mice. Oral presentation to American Endocrine Society 86th meeting New Orleans

Small GR, Dover AR, Hadoke PWF, Kenyon CJ, Seckl JR, Walker BR (2004) Angiogenesis is modulated by local regeneration of glucocorticoids by 11βHSD-1 in the vessel wall in vitro and in vivo in mice. Poster presentation to the British Atherosclerosis Society Spring Meeting

Small GR, Hadoke PWF, Dover AR, Sharif I, Gray GA, Kenyon CJ, Walker BR (2004) 11βHSD-1 -/- mice have enhanced angiogenesis and improved cardiac function following myocardial infarction. Hypertension 2004 Volume 44 Page 587 Poster presentation at 9th EECR meeting

Small GR, Hadoke PWF, Gray GA, Sharif I, Mullins JJ, Seckl JR, Walker BR (2004) 11βHSD1 -/- mice have enhanced angiogenic response and improved myocardial function following coronary artery ligation Circulation 110 (7) 266-266 Suppl. **Oral** presentation to AHA 2004

Small GR, Hadoke PWF, I Sharif, C Kenyon, Gray GA, Walker BR (2004) Glucocorticoids and Angiogenesis Association of Physicians Meeting Nottingham 2004 Oral Presentation

Small GR, Hadoke PWF,Sharif I, C Kenyon, Gray GA, Walker BR (2005) 11βHSD1 -/- mice have enhanced angiogenic response and improved myocardial function following coronary artery ligation Scottish Cardiac Society meeting 2005 Oral Presentation

Natalie ZM Homer, Gary R Small, Li Yin Ooi, Harsha Bollina, Rebecca M Reynolds, Brian R Walker, Ruth Andrew Quantitative analysis of mifepristone (RU38486) in plasma by HPLC triple quadrupole mass spectrometry British Endocrine Society Meeting 2005

Reviews

Hadoke PWF. , Macdonald L, Logie JJ, Small GR, Dover AR and Walker BR (2006) Intra-vascular glucocorticoid metabolism as a modulator of vascular structure and function Cellular and Molecular Life Sciences 63 (3) (Jan 16) 1-14.

Full papers

Small GR, Hadoke PWF, Sharif I, Dover AR, Armour D, Kenyon CJ, Gray GA, Walker BR (2005) Preventing regeneration of glucocorticoids by 11βhydroxysteroiddehydrogenase type 1 enhances angiogenesis and protects against ventricular dysfunction after myocardial infarction. PNAS 102 12165-121.

Chapter 1

Introduction

Introductory Remarks

Angiogenesis describes the proliferation, branching and remodelling of the existing vasculature to form new blood vessels. It is an essential component of vessel wall function and plays a key role in health and disease. New blood vessel formation in health is tightly regulated by local vessel wall and systemic factors (Conway *et al.* 2001).

Glucocorticoids although recognised as systemic mediators of vascular function through their effects on blood pressure, have received less attention as local regulators of vessel wall activity. Nevertheless the potential for these compounds to regulate vascular function locally has been demonstrated through their effects on mediators of vascular tone (Ullian 1999). Furthermore the local vascular effects of glucocorticoids may be influenced by the presence in the vessel wall of 11βHydroxysteroid dehydrogenase type 1 (11βHSD1) (Hadoke *et al.* 2001). 11βHSD1 amplifies local glucocorticoid concentrations by regenerating active glucocorticoids from inert substrates. Therefore either by altering systemic responses or via vessel wall 11βHSD activity; the potential exists for glucocorticoids to modulate angiogenesis.

A therapeutic target

Angiogenesis regulation has been identified as an attractive target for therapeutic intervention and both systemic and local factors have been used as potential angiogenic regulators (Jain 2003). Fibroblast derived growth factor (FGF) and vascular endothelial cell growth factor (VEGF) are mediators released by vessel wall constituents during new vessel formation and have been used individually to improve

ventricular revascularisation post myocardial infarction (Lopez *et al.* 1998; Iwakura *et al.* 2003). Systemic angiogenesis therapies have also been investigated in this setting; in particular stem cell therapy has been administered with some success in laboratory and clinical studies (Orlic *et al.* 2001; Rafii *et al.* 2002). Indeed myocardial revascularisation remains an attractive focus for angiogenesis research because of the clinical improvement seen with revascularisation therapy in ischaemic heart disease.

Stimulating angiogenesis for therapeutic benefit has also been explored in diabetic and peripheral vascular ulcer diseases when cutaneous wound healing is impaired (Graiani *et al.* 2004; Collinson & Donnelly 2004). The improvement in clinical and laboratory outcomes following angiogenesis induction in these conditions suggests an important role for angiogenesis in the healing response of the skin. Although increasing angiogenesis can be therapeutically attractive in some conditions there are other pathologies where the target for intervention has been the inhibition of excessive new vessel formation.

Limitation of the prolific angiogenesis in primary vascular tumours such as proliferating capillary haemangiomas has been a target of anti-angiogenic therapies. In malignancies of other tissues increased angiogenesis occurs and although in this situation the primary source of dysregulation and autonomous growth is not vascular in nature; excessive angiogenesis is nevertheless a major determinant of tumour size and haematogenous metastasis (Folkman 2001). Anti-angiogenic therapies have been used to inhibit tumour growth for example glucocorticoids are administered clinically in the treatment of proliferating capillary haemangiomas (Hasan *et al.* 2000). In addition recent clinical trials suggest that the anti-VEGF antibody

bevacizumab can safely and effectively be employed in colorectal cancer in combination with anticancer agents (Willett *et al.* 2004). Thus in vascular and non-vascular tumours anti-angiogenic therapy serves as an adjunctive therapy to traditional anti-cancer therapy.

Side effects of therapy

It would be unattractive however to maintain anti-angiogenic agents in the long term to prevent tumour progression since such agents would also delay tissue healing post surgical resection or have effects on co-incidental illnesses such as ischaemic heart disease. Furthermore the long-term effects of anti-angiogenic therapy on skeletal muscle remodelling and on the female reproductive cycle have not been studied. Thus although promising, the role of anti-angiogenic agents to treat tumour primary growth or prevent secondary metastatic spread requires further development.

In addition to the concerns regarding the long-term use of anti-angiogenic agents in tumour therapy other fears have arisen regarding the use of pro-angiogenic agents. There are hypothetical concerns that agents, which stimulate angiogenesis, could be tumorigenic. Furthermore in ischaemic heart disease, although angiogenic gene therapy with VEGF improved myocardial revascularisation there was also an increase in angiogenesis with atherosclerotic plaques (Schwarz *et al.* 2000). This gave rise to apprehensions regarding the possibility of plaque expansion, destabilisation and the development of acute coronary syndromes with pro-angiogenic therapy.

Future directions

Despite these initial setbacks modulation of angiogenesis by systemic and local factors continues to be a source of intense scientific and clinical research. Current emphasis has been to explore the possibility of targeting angiogenic modulators to the site of pathology whether by using endothelial cell markers for specific tissues or identifying specific angiogenic factors expressed in tumour cells (Sullivan & Bicknell 2003). Less attention has been directed at the important ability of the vessel wall to self regulate for example by modulating the effects of systemic angiogenesis factors. Therefore in this thesis we will study the regulation of angiogenesis by glucocorticoids and compare the effects of systemic glucocorticoids released by the adrenal cortex with the regeneration of glucocorticoids locally within the vessel wall by 11βHSD1. We will argue that local regulation of glucocorticoids concentrations by 11βHSD1 is an important determinant of angiogenesis responses. In particular we consider these effects in the contexts of myocardial revascularisation following infarction and in the cutaneous wound healing response.

This introductory chapter describes glucocorticoid physiology and examines how 11βHSDs modulate glucocorticoid action locally within tissues. The process of angiogenesis is detailed and the evidence for a modulatory role of glucocorticoids is discussed. Finally, a list of the aims of this thesis is presented.

Angiogenesis, the formation of new blood vessels from existing ones, is an essential component in the development of a vascular network but also plays a key role in the pathogenesis of many diseases (Conway *et al.* 2001). Clarification of the mechanisms that regulate angiogenesis is important for extending our understanding of the physiological control of new vessel growth and for developing tools for therapeutic manipulation of this process (Carmeliet & Jain 2000).

It is well established that angiogenesis can be inhibited by glucocorticoids (McNatt *et al.* 1999; Hasan *et al.* 2000; Hasan *et al.* 2003; Ingber *et al.* 1986; Hori *et al.* 1996; Folkman & Ingber 1987) (steroid hormones released from the adrenal cortex) but the mechanisms involved are unclear; possibilities include inhibition of inflammation, inhibition of vascular cell proliferation or migration. Many studies, however, have used pharmacological concentrations of glucocorticoids to demonstrate their angiostatic effects. Consequently, the influence of endogenous glucocorticoids, and their pre-receptor metabolism in target tissues, on angiogenesis has not been established. The presence of the enzyme 11β-hydroxysteroid dehydrogenase type 1 in the smooth muscle cells (Christy *et al.* 2003) suggests that glucocorticoids are generated from inert metabolites within the vascular wall itself. The work described in this thesis was designed to determine whether pre-receptor glucocorticoid generation in the vascular wall influences angiogenesis.

1.1 Glucocorticoids

Glucocorticoids (cortisol in man, corticosterone in rodents) are known therapeutically for their anti-inflammatory and immunosuppressive actions (Barnes 1998) but have much wider-ranging effects on body physiology. These are most clearly demonstrated in the clinical syndromes of Cushing's or Addison's diseases (Addison 1855) where altered glucocorticoid availability creates a diffuse clinical picture involving many organ systems.

1.1.2 Steroid Hormone Structure

Glucocorticoids, in common with other steroid hormones, contain a common backbone molecule with three 6-carbon hexane rings and a single 5-carbon pentane ring. Carbon atoms within steroids are numbered starting in the A ring and steroids with a ketone group at C-11 are known as 11-ketosteroids (Figure 1.1).

1.1.3 Biosynthesis

Glucocorticoids are synthesised, from cholesterol, in the inner zones of the adrenal cortex (predominantly in the zona fasciculata, with a smaller contribution from the zona reticularis). Adrenal hormone production is catalysed by members of the CYP oxidative enzyme family (previously labelled cytochrome P_{450}) located in the membranes of the mitochondria, cytosolic microsomes and endoplasmic reticulum (ER).

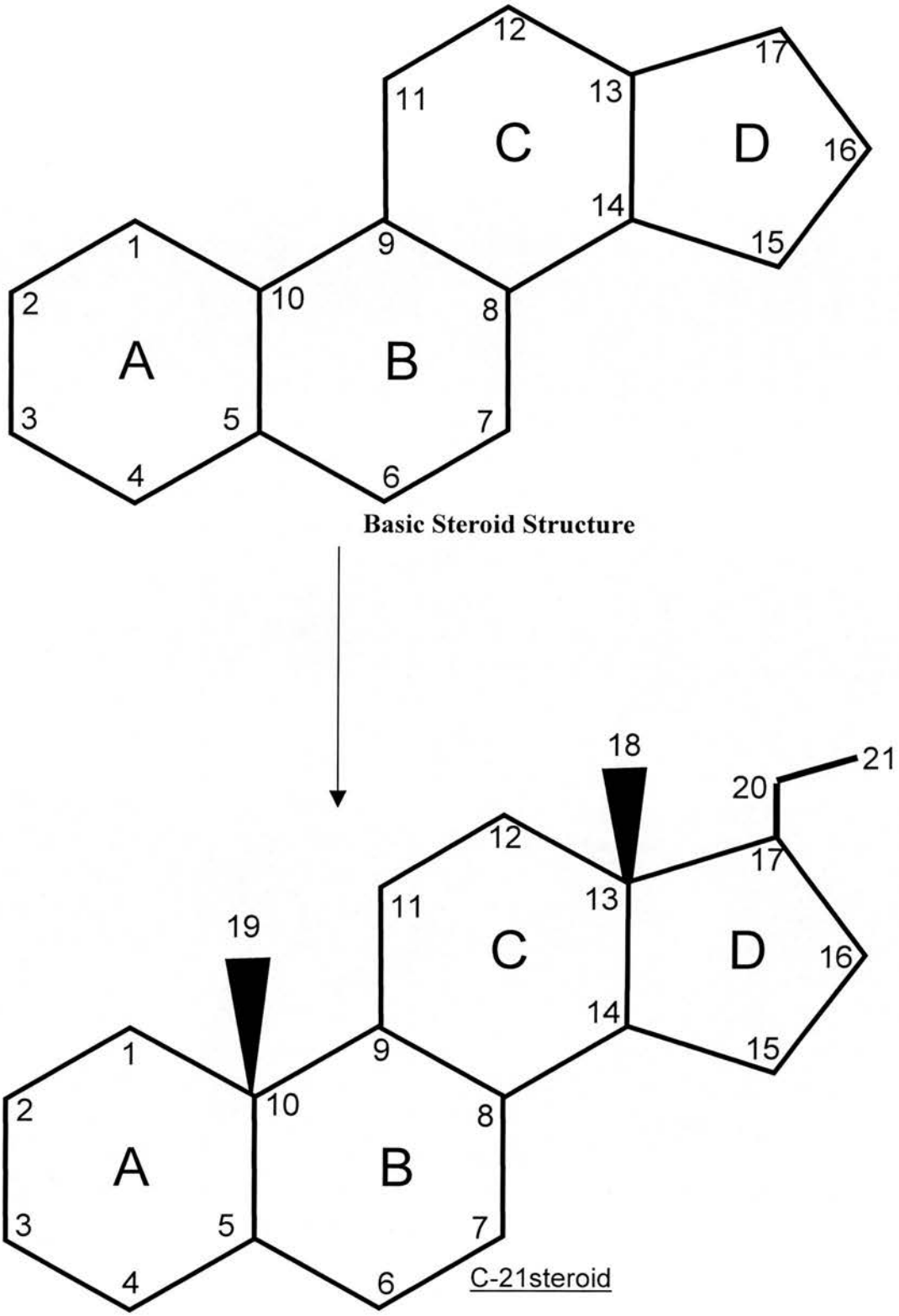


Figure 1.1 Basic Steroid Structure

The carbon atoms are numbered in a sequence commencing with ring A. C_{21} steroids have either glucocorticoids or mineralocorticoid properties.

1.1.4 Secretion

Glucocorticoid synthesis by the adrenal cortex is controlled by hormonal interactions among the hypothalamus, pituitary and adrenal glands (Jacobson & Sapolsky 1991; Jacobson 2005). Intra-adrenal glucocorticoid storage is minimal thus biosynthesis is closely associated with adrenal secretion.

Neural stimulation of the hypothalamus (e.g. in response to stress) causes the release of corticotrophin releasing hormone (CRH) from hypothalamic neurons into the hypothalamic-hypophyseal portal system and thence to the pituitary. At the pituitary, stimulation of CRH receptors on corticotrophs of the anterior pituitary results in the rapid release of adrenocorticotrophic hormone (ACTH) into the systemic circulation (Jacobson & Sapolsky 1991; Jacobson 2005).

ACTH is synthesised as part of the precursor polypeptide pro-opiomelanocortin (POMC) and acts via plasma membrane receptors (melanocortin-2-receptors) on adrenocortical cells, to stimulate steroidogenesis in an acute and chronic fashion (Clark *et al.* 2003; Beuschlein *et al.* 2001). Acutely, ACTH increases the conversion of cholesterol to pregnenolone; this effect is mediated by activation of cholesterol delivery to the inner mitochondrial membrane. Chronically ACTH increases the expression and activities of most of the enzymes of the steroidogenic pathway and can also stimulate adrenal hyperplasia and hypertrophy.

Glucocorticoids are also secreted in a diurnal fashion in response to a circadian stimulation of ACTH release (Jacobson & Sapolsky 1991; Jacobson 2005). Circadian rhythm is usually synchronised to sleep-wake cycles; thus glucocorticoid secretion peaks 2-4 hours before awakening, and then decreases to almost zero just before sleep. ACTH is secreted in brief pulses that raise plasma ACTH and glucocorticoids: plasma glucocorticoids stay elevated longer than ACTH because of slower clearance.

ACTH secretion is further regulated by glucocorticoids via a negative feedback loop (Aguilera *et al.* 2001; Jacobson & Sapolsky 1991; Jacobson 2005). Feedback occurs at both pituitary and hypothalamic levels. ACTH secretion is suppressed at the hypothalamus by inhibiting CRH synthesis and release, and at the level of the pituitary by inhibiting CRH receptor expression, ACTH secretion and POMC transcription. Glucocorticoids also affect ACTH secretion indirectly by blocking the stimulatory effects of CRH on POMC transcription and ACTH release.

1.1.5 Glucocorticoids in plasma

Glucocorticoids are transported in plasma bound to corticosteroid-binding protein (CBG) and albumin, with only 5-10% unbound in the plasma (Hammond *et al.* 1990). Protein binding improves the transport of glucocorticoids (which are insoluble in water) in the plasma and provides a reservoir that buffers free hormone concentrations.

1.1.6 Metabolism

Inactivation of glucocorticoids occurs largely by metabolism within the liver followed by conjugation and excretion in the urine. There are five possible initial fates for cortisol/ corticosterone in primary inactivation reactions (Figure 1.2):

1. Hepatic reduction of the Δ^4 double bond of the A-ring by 5α- or 5β-reductases; this is the major route of glucocorticoid metabolism. Subsequent hepatic reduction by 3α-hydroxysteroid dehydrogenase (3α-HSD) yields tetrahydro-metabolites.
2. Reduction by 20α- and 20β-hydroxysteroid dehydrogenases to produce 20-hydroxy derivatives. These enzymes can also reduce tetrahydro-derivatives further to yield cortols and cortolones.
3. Oxidative side chain removal produces 19-carbon steroids with a 17-ketone group (e.g. 11-Hydroxyandrostenedione from cortisol).
4. Hepatic hydroxylation to produce 6β-hydroxy cortisol (corticosterone).
5. Interconversion of corticosterone (cortisol) with its inert 11-keto-metabolite 11-dehydrocorticosterone (cortisone) by the 11β-HSD isozymes.

Further transformations of metabolites are required to improve their solubility in water and, therefore, they undergo several additional modifications, including: reduction, oxidation, hydroxylation and conjugation. Hepatic metabolism of glucocorticoids can be influenced by hormones (e.g. thyroxine), age (metabolism declines with age) inter-current illness, obesity and drugs (e.g. phenytoin, rifampicin).

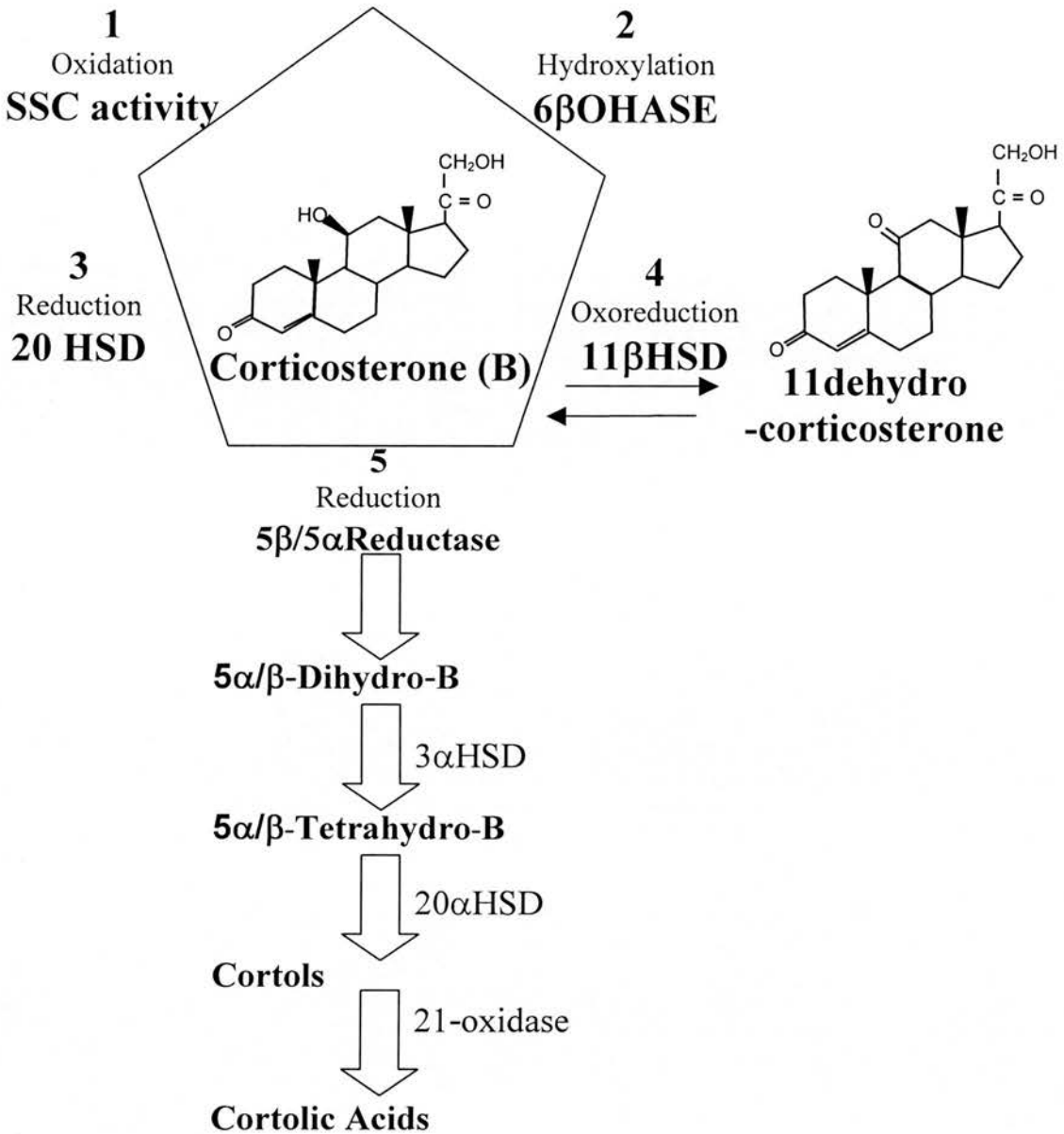


Figure 1.2 Hepatic Metabolism of Glucocorticoids

There are five initial fates for corticosterone (B) upon hepatic metabolism. The predominant mode of inactivation is by 5β/5α reductase. SCC, side chain cleavage; 6βOHASE, 6β-hydroxylase; 20HSD, 20α hydroxysteroid dehydrogenase; 3αHSD, 3α hydroxysteroid dehydrogenase; 11βHSD 11βhydroxysteroid dehydrogenase.

1.1.7 Action of Glucocorticoids

Glucocorticoids, as members of the steroid family of hormones, exert their effects predominantly by binding to and activating specific transcription factors that alter the expression of several genes. Glucocorticoids probably enter target cells by passive diffusion of the free hormone (although possible transport systems have been described) (Lan *et al.* 1984). Once inside the cell the steroid binds to and activates intracellular receptors, either glucocorticoid or mineralocorticoid receptors. For the remainder of this discussion we will consider the action of glucocorticoids at glucocorticoid receptors.

The glucocorticoid receptor has splice variants; GRα and GRβ. GRβ is believed to act as a dominant negative inhibitor of GRα action (Oakley *et al.* 1996) but this is not expressed at significant levels in most species and tissues studied. The inactive receptor complex consists of GR bound to 2 molecules of heat shock protein 90 (hsp90), a 59kDa immunophilin protein (hsp59) and other inhibitory proteins (CyP40) and a 23kd acidic protein) (Pratt 1993). The hsp90 molecules act as molecular chaperones preventing the unoccupied GR localising to the nucleus. Upon corticosterone binding, the inhibitory heat shock proteins are displaced from the receptor, thus allowing translocation of the ligand-receptor complex to the nuclear compartment. Dimerisation of GR-ligand complexes in the nucleus permits binding of GR to specific palindromic DNA consensus sequences termed glucocorticoid response elements (GRE) that are found in the 5'-upstream promoter region of glucocorticoid-responsive genes. Gene transcription may be activated or repressed by

GR binding depending on the availability and mode of action of other transcription factors.

Glucocorticoids may also have non-genomic effects that are further sub-classified as either specific or unspecific (Buttgereit 2000). Specific non-genomic effects on second messenger systems are considered to be mediated by steroid selective membrane receptors or via CBG activation of adenylate cyclase. Glucocorticoid binding sites have been described in rat neuronal membranes (Liposits & Bohn 1993) human leukaemic cells (Gametchu *et al.* 1999) and human endothelial cells (Moghadam et al 2002).

Unspecific non-genomic effects occur at high glucocorticoid concentrations and are believed to result from direct interactions with biological membranes that affect the activities of membrane-associated proteins (Buttgereit 2000). High concentrations of methyl prednisolone for example are observed to inhibit cation cycling in activated thymocytes with little effect on protein synthesis and independent of reductions in ATP (Buttgereit 2000). The physiological relevance and clinical significance of these non-genomic actions remain unclear.

1.1.8 Effects of Glucocorticoids

Glucocorticoids serve the purpose of basal regulation of metabolic processes and adjustment of these processes to stresses such as injury and infection (Barnes 1998). During these stresses, increased GCs liberate fuels, divert energy from unhelpful processes such as growth and reproduction, protect against cardiovascular shock, and

provide the crucial brake to prevent bystander damage from the innate and adaptive immune responses. In their latter role, GCs have many anti-proliferative effects (Longenecker *et al.* 1984). If sustained in the longer term, these useful short-term adaptations become maladaptive, resulting for example in the features of Cushing's syndrome (Cushing 1912). These features include adverse cardiovascular effects (Walker & Williams 1992) and poor wound healing (Gordon *et al.* 1994). In this context, glucocorticoids have been shown to inhibit angiogenesis (Folkman *et al.* 1983). A recent key development in our understanding of glucocorticoid physiology has been the recognition that the effects of glucocorticoids are modulated within each target tissue by enzymes which control the balance between inert and active steroids (Seckl & Walker 2001). These 11βHSDs have been demonstrated in the vessel wall and, crucially, appear to increase local GC action in inflamed or injured tissues (Cai *et al.* 2001; Christy *et al.* 2003; Hadoke *et al.* 2001). Manipulation of these enzymes may offer an approach to manipulate angiogenic responses in a tissue-selective way, without preventing the physiological effects of glucocorticoids in other tissues.

1.1.8.1 Effects on the Cardiovascular System

Glucocorticoids may influence many aspects of vascular function including: vascular tone, haemostasis, vessel wall inflammatory responses and angiogenesis. Many of these effects have been observed in the presence of large concentrations of glucocorticoids and the question of whether they have physiological relevance has received little attention. There is however, evidence from clinical conditions of altered glucocorticoid secretion to suggest that endogenous concentrations of glucocorticoids alter vascular tone (Whitworth 1994).

Blood pressure- Classically glucocorticoids were thought to cause hypertension as a result of salt and water retention in the kidneys. Although these hormones can directly influence sodium handling and are important for the regulation of angiotensinogen, AVP and atrial naturetic peptide (ANP) (Montrella-Waybill *et al.* 1991; Raff 1987)) (Shields *et al.* 1988) – each of which can effect renal sodium/ water handling. It is now also appreciated that glucocorticoids directly affect vascular tone (Whitworth 1994; Ullian 1999).

Vascular Tone- Although their exact cellular locations remain controversial both glucocorticoid and mineralocorticoid receptors are found in the vessel wall (Ullian 1999). Glucocorticoids increase vascular contractility to several agonists, including noradrenaline and angiotensin II, by increasing receptor expression and improving coupling of second messenger systems (Ullian 1999; Yang & Zhang 2004). Endothelium-dependent relaxation may also be impaired by glucocorticoids since nitric oxide production is reduced in endothelial cells cultured in the presence of glucocorticoids and a number of mechanisms have been proposed including alterations in L-arginine availability or transport, endothelial nitric oxide synthase down regulation and reduced co-factor availability (Simmons *et al.* 1996; Whitworth *et al.* 2002; Christy *et al.* 2003; Rees *et al.* 1990; Radomski *et al.* 1990).

Coagulation and Fibrinolysis- Patients with Cushing's disease are recognised to have an increased risk of pulmonary embolism and have been found to have abnormally high levels of both liver (for example XII, XI, IX, VIII) and endothelial

cell (Von Willebrand Factor and plasminogen activator inhibitor-1) synthesised haemostatic factors (Patrassi *et al.* 1985; Jacoby *et al.* 2001; Casonato *et al.* 1999). However it is unclear whether the alterations in haemostasis in Cushing's disease reflects a direct effect on coagulation/fibrinolysis factors or whether the observed changes in fact reflect other aspects of the condition for example insulin resistance and hypertension- both of which might be expected to alter endothelial cell function. To date attempts using cell culture techniques *in vitro* to dissect the potential mechanisms of glucocorticoids to alter haemostasis have used large, supra-physiological concentrations of glucocorticoids and thus the physiological relevance of these findings is difficult to interpret (Yamamoto *et al.* 2004).

Vascular Inflammation- Vessel wall inflammation contributes to the formation, growth and complications of atherosclerotic plaques but also influences vessel remodelling following angioplasty. The effects of glucocorticoids on vessel inflammation have been studied in both of these situations.

Animal data examining the influence of glucocorticoids on vascular remodelling post intraluminal injury is difficult to interpret, some groups have found benefit from glucocorticoid coated stents in reducing neointimal hyperplasia and in-stent restenosis, others have found no effect (Villa *et al.* 1994; Strecker *et al.* 1998). The differences in outcome may reflect the differences in vessels injured, the species used and differences in the pharmacology of the stent and eluting agents.

In clinical trials adjuvant systemic prednisolone, given to patients undergoing coronary artery stenting, decreased neointimal lesions and restenosis (Versaci *et al.* 2002). The mechanism of glucocorticoid action in preventing neointimal hyperplasia

and/or vessel recoil is uncertain. Intimal anti-inflammatory effects would be expected to play a role but glucocorticoid interactions in the adventitia may also be important.

Angiogenesis- Clinically, large doses of glucocorticoids have been used to inhibit angiogenesis in vascular tumours; for example, in the treatment of proliferating capillary haemangiomas (Hasan *et al.* 2000; Hasan *et al.* 2003). The mechanism of glucocorticoid-induced angiostasis is unclear but may involve anti-inflammatory, anti-proliferative and anti-migratory influences (Folkman & Ingber 1987). As will be described below, this thesis focuses on the anti-angiogenic effects of glucocorticoids. These are reviewed in detail in section 1.4. A major question was the possible role of 11βHSDs in modulating glucocorticoid actions within the vessel wall, for which a fundamental review of the role of 11βHSDs is required.

1.2 11βHydroxysteroid Dehydrogenases

The isozymes of 11βHSD catalyse inter-conversion of active glucocorticoids and their inert 11-keto metabolites. This provides a mechanism for pre-receptor metabolism of glucocorticoid within target cells. Thus, these enzymes have the potential to alter local glucocorticoid levels without altering systemic concentrations and may significantly influence tissue metabolic, immune, and maturation responses.

1.2.1 Physiological Relevance of 11βHSDs

11βHSDs were initially thought to be a single enzyme, involved in the clearance of glucocorticoids (Amelung *et al.* 1953). More recently it was appreciated that within mineralocorticoid target tissues (for example the distal nephron) 11βHSD

metabolism of glucocorticoids also has a significant physiological role (Arriza *et al.* 1987). In these tissues 11βHSD metabolism of glucocorticoids acts as a barrier to prevent glucocorticoids accessing the mineralocorticoid receptor. This is important since physiologically glucocorticoids are found in 100-1000 fold excess of mineralocorticoids and mineralocorticoid receptors are able to bind glucocorticoids and mineralocorticoids with comparable affinity (Arriza *et al.* 1987). Thus to allow mineralocorticoids access to their receptors it is necessary for 11βHSD to metabolise glucocorticoids.

For example the importance of 11βHSD enzyme activity in the distal nephron was demonstrated by the Syndrome of Apparent Mineralocorticoid excess (SAME) (Ulick *et al.* 1979; Stewart *et al.* 1988). This rare, autosomal recessive, congenital disorder presents with signs and plasma biochemistry characteristics of excessive activation of mineralocorticoid receptors, including severe hypertension, low plasma renin and hypokalaemia despite low plasma aldosterone levels. Furthermore patients with SAME have high concentrations of urinary cortisol metabolites (tetrahydrocortisols and cortols) in comparison to those of cortisone (tetrahydrocortisone and cortolones) (Ulick *et al.* 1979) suggesting that altered 11βHSD glucocorticoid inactivitation in the distal nephron might be responsible for the syndrome.

A condition similar to SAME was also observed in patients prescribed carbenoxolone (active ingredient: a hemi-succinate derivative of glycyrrhetinic acid) or following excessive consumption of liquorice (contains glycyrrhetinic acid) (Epstein *et al.* 1977). Administration of dexamethasone or the mineralocorticoid receptor (MR) antagonist spironolactone ameliorated the signs of liquorice-induced

mineralocorticoid excess (Shackleton *et al.* 1980; Doll *et al.* 1968; Hoefnagels & Kloppenborg 1983) whilst physiological doses of cortisol exacerbated the features of SAME (Oberfield *et al.* 1983) suggesting that an ACTH-dependent adrenal MR agonist was responsible for these syndromes. This was supported by earlier findings that had demonstrated liquorice-induced mineralocorticoid excess to be dependent upon intact adrenal function (Borst *et al.* 1953).

Confirmation that the mode of action of liquorice was to inhibit 11βHSD activity (Stewart *et al.* 1987; MacKenzie *et al.* 1990) explained how MR could be selective in the presence of both aldosterone and cortisol and led to the description of the mechanism involved in SAME (Stewart *et al.* 1996). Hence, it was proposed that the physiological role of 11βHSD was to protect MR from illicit activation by glucocorticoids and allow them to bind aldosterone.

Further evidence for the influence of 11βHSD2 in mineralocorticoid target tissues has been obtained through the development of 11βHSD2 null (-/-) transgenic mice. Transgenic deficiency of 11βHSD2 in mice results in a collection of physiological findings similar to SAME with severe hypertension and hypokalaemia (Kotelevtsev *et al.* 1999).

11βHSD2 is also expressed at high levels in sites where mineralocorticoid receptors are not abundant but a barrier to glucocorticoids is still required: for example, in the placenta where 11βHSD2 activity is believed to protect the foetus from deleterious effects of maternal corticosteroids (Brown *et al.* 1996a). This is supported by findings in neonatal rats where foetal exposure to dexamethasone (a poor substrate for 11βHSD2) or inhibition of 11βHSD2 by carbenoxolone during pregnancy, reduces birth weight (Benediktsson *et al.* 1993; Lindsay *et al.* 1993). Furthermore

administration of 11βHSD inhibitors to pregnant rats produces effects in the offspring that are consistent with increased glucocorticoid exposure in utero including growth retardation and subsequent hypertension and glucose intolerance (Nyirenda *et al.* 1998). Thus through these and other studies the prominence of 11βHSD has altered from an obscure route of glucocorticoid catabolism to an enzyme with significant physiological function and important effects on glucocorticoid activity.

1.2.2 Two isozymes: 11βHSD Type1 and 2

The existence of two isozymes of 11βHSD was suggested from evidence in SAME patients since these individuals had lost the ability to metabolise cortisol to cortisone yet were still able to convert oral cortisone to cortisol (Ulick *et al.* 1979; Stewart *et al.* 1988). In addition, with the cloning of the enzyme from rat liver (Agarwal *et al.* 1989) it became clear that 11βHSD enzyme activities in liver and kidney had different kinetic, regulatory and immunohistochemistry characteristics. Liver 11βHSD– termed 11βHSD1 from this point forward – is a low affinity NADP(H)-dependent enzyme and is expressed in a wide range of tissues including the liver, lung, adipose tissue, gonads and the CNS (Krozowski *et al.* 1990). Kidney 11βHSD enzyme - named 11βHSD2 - is a high affinity, NAD-dependent enzyme. The limited tissue distribution of 11βHSD2 is consistent with a role in MR protection; it is expressed in renal cortical collecting tubules, the distal colon, sweat glands and the placenta (Agarwal *et al.* 1994) (Brown *et al.* 1996a).

1.2.3 11βHSD directionality: reductase, dehydrogenase or both

In initial studies 11βHSD1 in the rat liver was found to have both reductase and dehydrogenase activities although the reductase activity was unstable *in vitro* (Seckl & Walker 2001). Subsequently it has been demonstrated that 11βHSD1 functions predominantly (if not exclusively) as a reductase *in vivo* and in intact cell systems, converting inactive 11-keto metabolites into active glucocorticocoids (Rajan *et al.* 1996; Kotelevtsev *et al.* 1997; Jamieson *et al.* 1995; Jamieson *et al.* 2000). There are, however, a few studies where 11βHSD1 dehydrogenase activity has been reported in intact cell preparations, with the direction of 11βHSD1 activity dependent upon physiological or developmental status of the particular cell type. In human omental adipose stromal cells, 11βHSD1 changes from a dehydrogenase to a reductase when these cells differentiate into adipocytes and in Leydig and neuronal cells, both 11βHSD–1 reductase and dehydrogenase activities have been reported (Bujalska *et al.* 2002; Gao *et al.* 1997).

Whenever cells are disrupted, however, or the enzyme purified, reductase activity is lost. It has been postulated that the change in directionality between intact cells and homogenates reflects the intracellular localization of 11βHSD1 within the lumen of the endoplasmic reticulum (ER), where neighbouring enzymes may be powerful generators of the reduced co-substrate NADP phosphate (NADPH) (Seckl & Walker 2001). Upon addition of an NADPH regenerating system employing the cytosolic enzyme glucose-6-phosphate dehydrogenase it is possible to regain reductase activity from tissue homogenates and purified enzyme (Walker *et al.* 2001; Agarwal *et al.* 1990), implying that reductase activity predominates in intact cells as a consequence of large concentrations of NADPH within the ER lumen. The enzyme hexose- 6-

phosphate dehydrogenase (H6PDH) has recently been shown to serve this crucial role in generating NADPH levels in the ER (Draper *et al.* 2003).

In contrast, 11βHSD2 exclusively catalyses the dehydrogenation of 11β-hydroxyglucocorticoids, has a nanomolar K_m for glucocorticoids, utilizes NAD^+ as a cofactor, and is localized in the endoplasmic reticulum membrane with a cytoplasmic orientation of its catalytic domain (Albiston *et al.* 1994; Brown *et al.* 1993; Stewart *et al.* 1994; Kataoka *et al.* 2002). The enzyme exhibits cell-specific expression in mineralocorticoid target tissues, such as epithelial cells from colon or renal cortical collecting tubules, where its main function is to protect the non-selective mineralocorticoid receptor from activation by glucocorticoids (Stewart *et al.* 1995).

1.2.4 Regulation of 11βHSDs

The complex tissue- and species-specific biology of 11βHSD1 and 11βHSD2 indicates that the expression of these isozymes is regulated rather than constitutive (Moisan *et al.* 1992b; Yang *et al.* 1992; Brown *et al.* 1996b). Numerous factors have been shown to regulate 11βHSD 1 expression and there is increasing evidence that 11βHSD 2 expression is also regulated (Lanz *et al.* 2001; Alikhani-Koopaei *et al.* 2004; Heiniger *et al.* 2003).

The mechanisms controlling tissue- and species-specific regulation of 11βHSD expression are not fully understood. Analysis of the rat 11β-HSD 1 gene transcripts suggested tissue-specific differential promoter usage in liver and kidney (Moisan *et al.* 1992a), which is possibly due to the differential tissue expression of regulatory/ transcription factors. Furthermore the promoter region of the human 11βHSD2 gene

has also been examined and an inverse correlation between promoter methylation and 11βHSD2 expression is apparent when placenta, skeletal muscles, liver, lung, and renal proximal and distal tubules are compared (Alikhani-Koopaei *et al.* 2004). Altered transcription factor binding to methylated promoter regions (e.g. as observed with Sp1/Sp3) could be a mechanism for these observed differences.

Several initiating factors that trigger alterations in the expression and activity of 11βHSD1 and 2 have also been identified. Enhanced expression of 11βHSD2 has been observed with the transcription factor nuclear factor I (NFI) and decreased 11βHSD2 expression has been observed with various stimuli including: hypoxia (Heiniger *et al.* 2003), shear stress (Lanz *et al.* 2001), angiotensin II (Lanz *et al.* 2003) and TNF-α (Heiniger *et al.* 2001). The corresponding signal transcription pathways and some relevant transcription factors have been identified (Heiniger *et al.* 2003). In addition reduced 11βHSD2 activity has been linked to renal sodium retention in liver cirrhosis (Escher *et al.* 1998), nephrotic syndrome (Kerstens *et al.* 2003) and hypoxia (Heiniger *et al.* 2003).

11βHSD1 has a broader tissue expression than 11βHSD2 and the factors that regulate expression and activity of 11βHSD1 appear to possess tissue and species specificity. Variations in 11βHSD 1 expression within tissues and between species are observed with sex steroids, insulin, and thyroid hormone (Seckl & Walker 2001; Tomlinson *et al.* 2004b; Sandeep & Walker 2001).

In general, 11βHSD1 activity and expression is lower in tissues from female compared with male animals. The sexual dimorphism of 11βHSD1 expression is mediated by the influence of oestrogen on growth hormone (GH) secretory patterns

(Low *et al.* 1994a). Upon reaching sexual maturity, oestrogen in females increases the frequency of GH pulsatile secretion (Lanz *et al.* 2003). This results in greater constant circulating GH concentrations in females compared to males (Eden 1979) and the elevated GH in females reduces 11βHSD1 activity and expression (Painson *et al.* 1992; Smith & Funder 1991).

There are data to suggest that other hormones may regulate 11βHSD1 expression however the effects of these hormones are inconsistent and are influenced by experimental conditions and appear to be tissue dependent (Tomlinson *et al.* 2004b). For example insulin represses expression in rat hepatocytes *in vivo* (Liu *et al.* 1996)and human skin fibroblasts *in vitro* (Hammami & Siiteri 1991) yet had no effect on human omental preadipocytes (Bujalska *et al.* 1999). Thyroid hormone decreases rat pituitary 11βHSD1 activity (Whorwood *et al.* 1993) but the opposite effect was seen in rat testis (Nwe *et al.* 1997). And thyroid hormone in rats decreases hepatic 11βHSD1 activity *in vivo* (Whorwood *et al.* 1993), but not hepatocyte activity *in vitro* (Ricketts *et al.* 1998).

More consistent regulatory influences across species and tissues are observed with glucocorticoids, inflammatory cytokines and leptin (Tomlinson *et al.* 2004b). Glucocorticoids increase 11βHSD1 expression or activity both in human fibroblast cell cultures and in rat vascular smooth muscle cells and hepatocytes *in vitro* (Hammami & Siiteri 1991; Jamieson *et al.* 1999; Takeda *et al.* 1994a). *In vivo*, glucocorticoids induce 11βHSD1 in liver and hippocampus, whist adrenalectomy decreases hepatic 11βHSD1 mRNA and activity (Low *et al.* 1994b; Walker *et al.* 1994b).

Inflammatory cytokines are known to increase the activity and expression of 11βHSD 1 *in vitro*. Tumor necrosis factor-α (TNF-α) increases renal 11βHSD1 activity (Escher *et al.* 1997), interleukins increase 11βHSD1 activity in granulosa cells (Evagelatou *et al.* 1997), and interleukin1β and TNF-α increase 11βHSD1 expression in vascular smooth muscle cells (Cai *et al.* 2001).

Leptin has also been shown to increase 11βHSD1 activity and expression *in vitro* and i*n vivo. In vivo* in mouse liver leptin induced 11βHSD1 expression (Liu *et al.* 2003). *In vitro* human omental preadipocytes and mouse hepatocytes both exhibited increases in 11βHSD1 expression and activity in the presence of leptin (Tomlinson *et al.* 2001; Liu *et al.* 2003).

There are many other single studies examining the effects of different factors on 11βHSD1 expression and/or activity for example PPAR α or γ agonists, protease inhibitors and others (Tomlinson *et al.* 2004b). The tissue and species specificity of 11βHSD1 responses however suggests that these results should be corroborated with further investigations to determine the physiological and clinical influence of the factors used.

1.2.5 11βHSD 1 modulation of glucocorticoid activity

Local tissue expression of 11βHSD1 creates the possibility that within the local environment of enzyme expression glucocorticoid levels may fluctuate without overtly effecting systemic glucocorticoid concentrations. 11βHSD1 thus is a pre-receptor determinant of glucocorticoid action and may influence a broad range of

glucocorticoid actions from tissue energy metabolism to tissue maturation and growth responses (Tomlinson *et al.* 2004b).

Amplification of local glucocorticoid concentrations is, in part, conditional on the availability of 11βHSD1 substrate. The main source for 11keto steroids (cortisone or 11dehydrocorticosterone) is 11βHSD2 in the kidney. In humans, cortisone circulates in the plasma largely unbound to plasma proteins at concentrations of 50-100nmol/L without a pronounced diurnal variation (Seckl & Walker 2001). However in the mouse plasma 11dehydrocorticosterone levels are lower at 3-5nmol/L as a consequence of increased clearance (Seckl & Walker 2001). In comparison to cortisone, 95% of cortisol is bound to plasma proteins such that free plasma cortisol levels are around 1-100nmol/L. Therefore circulating cortisone levels are in excess of, or at least at parity with, cortisol levels for some (if not all) of the day (Seckl & Walker 2001).

11βHSD1 has been shown to amplify the effects of glucocorticoids by local regeneration and evidence for this comes largely from studies on the liver. Physiologically, in hepatocytes, glucocorticoids oppose the effects of insulin and increase glucose production; for example, by up regulating key gluconeogenic enzymes such as PEPCK. Clinical studies with non-selective inhibitors of 11βHSD demonstrate a reduction in hepatic glucocorticoid effects and improved insulin sensitivity (Walker *et al.* 1995). Furthermore, in 11βHSD1 deficient transgenic mice glucocorticoid antagonism of insulin is attenuated resulting in blunted hyperglycaemia during stress or high fat feeding (Kotelevtsev *et al.* 1997). The influence of *amplified* hepatic glucocorticoid concentrations has been studied in transgenic mice over expressing hepatic 11βHSD1. These animals demonstrate

insulin resistance as well as dyslipidaemia and hypertension (Paterson *et al.* 2004), thus indicating that amplification of local glucocorticoid concentrations within the liver by 11βHSD1 can antagonise the effects of insulin and modulate fuel metabolism.

11βHSD1 is also expressed in adipose tissue where it functions predominantly as a reductase (Napolitano *et al.* 1998) and may also antagonise the effects of insulin and contribute to the metabolic syndrome. In clinical and animal studies of obesity, adipose 11βHSD1 is dysregulated (Wake & Walker 2004) and it is hypothesised that 11βHSD 1 plays a role in the development of central obesity (Wake & Walker 2004; Livingstone *et al.* 2000b; Masuzaki *et al.* 2001; Morton *et al.* 2001; Rask *et al.* 2002). There are however studies examining 11βHSD1 expression in rodent models and 11βHSD1 activity in human studies that have shown none or converse changes in 11βHSD1 in obesity (Tomlinson *et al.* 2001; Tomlinson *et al.* 2004a; Tomlinson *et al.* 2004b). The apparent discrepancies of these data may reflect the different techniques and assays to determine 11βHSD1 activity or the small sample sizes in the clinical studies.

Glucocorticoids also modulate developmental, metabolic and neuronal signalling within the brain via both GR and MR (McEwen *et al.* 1986). Since 11βHSD1 is expressed in cerebellum, pituitary and hippocampal neurones and other regions of the CNS (Moisan *et al.* 1992b; Sakai *et al.* 1992) investigators have examined the influence of local glucocorticoid regeneration at these sites. *In vitro* in hippocampal neurones 11βHSD1 acts as a reductase to amplify glucocorticoid concentrations and in 11βHSD1 knockout mice the absence of 11βHSD1 in these cells decreases corticosterone levels within the hippocampus and appears to protect from age-related

learning impairment (Yau *et al.* 2001). Other brain centres altered in 11βHSD1 knockout mice include the HPA axis. 11βHSD1 knockout mice exhibit abnormalities of the HPA axis with elevated corticosterone and ACTH levels, enhanced responses to stress, and insensitivity of HPA axis suppression with exogenous cortisol (Kotelevtsev *et al.* 1997; Harris *et al.* 2001).

11βHSD1 is also expressed in human bone and primary osteoblast cultures (Cooper *et al.* 2000) and it functions primarily as a reductase to amplify local glucocorticoid concentrations. From clinical studies it would appear that 11βHSD1 influences bone remodelling; inhibition of 11βHSD activities with carbenoxolone had no impact on bone formation but did result in suppression of bone resorption (Cooper *et al.* 2000).

11βHSD1 is expressed in human and rodent ovaries although the function of the enzyme at this site is undetermined (Thomas *et al.* 1998; Tetsuka *et al.* 1999; Hillier & Tetsuka 1998). A switch from 11βHSD type 2 to type 1 isozyme expression is thought to account for the increase in intra follicular cortisol concentration after the LH surge prior to ovulation (Hillier & Tetsuka 1998). The increase in active glucocorticoid may serve to mature the developing oocyte and/or limit the inflammatory wound healing response of the ovary. In the mouse the relevance of ovarian 11βHSD1 remains unclear since 11βHSD1 transgenic deletion does not impair reproduction and ovarian function appears to be intact.

11βHSD1 and 2 are also expressed in the blood vessel wall and with the development of transgenic mice deficient in either 11βHSD1 or 11βHSD2 further in sight has been gained as to the vascular functions of these isozymes (Christy *et al.*

2003; Hadoke *et al.* 2001). Modulation of vascular function by 11βHSD will be discussed in the following section.

1.2.6 Vascular 11β-HSD1 Activity and Expression

Vascular 11βHSD activity was first described in rabbit aorta and rat mesenteric arteries (Kornel *et al.* 1982; Funder *et al.* 1989) and has subsequently been investigated in vessels and cultured vascular cells from several species (Hatakeyama *et al.* 1999; Hadoke *et al.* 2001; Walker & Williams 1992)). Localisation studies have examined the location of 11βHSD expression within the vessel wall by RT-PCR and identified the 11βHSD1 isozyme in mouse aortic vascular smooth muscle cells (VSMC) (Christy *et al.* 2003). This corroborated findings from earlier studies that had examined rat mesenteric and aortic vessels and found 11βHSD1 expression within VSMC (Takeda *et al.* 1994b; Brem *et al.* 1995; Walker *et al.* 1991; Brem *et al.* 1998). Immunohistochemical studies of rat vessels have suggested this isozyme is also expressed in adventitial fibroblasts (Brereton *et al.* 2001).

In VSMC from mouse aorta 11βHSD2 mRNA was not identified by RT-PCR studies (Christy *et al.* 2003). However it appears from other localisation studies that 11βHSD2 expression in VSMC could be species and vascular bed specific (Brem *et al.* 1998; Hatakeyama *et al.* 1999; Smith *et al.* 1996; Kyossev *et al.* 1996).

The expression of 11βHSD isozymes is less clearly defined in endothelial cells. Recent evidence from RT-PCR studies using intact mouse aorta suggested that 11βHSD type 2 and not type 1 expression is localised to the endothelium (Christy *et al.* 2003). However data from other studies are not in complete accordance with these

findings and suggest that there are species and specific vascular bed differences in isozyme endothelial expression. RT-PCR and activity studies indicate that both 11βHSD1 and 11βHSD2 are expressed in rat aortic endothelial cells (Brem *et al.* 1998), yet *in situ* hybridisation and immunohistochemistry did not identify 11βHSD1 endothelial cell-specific expression of this isozyme (Walker *et al.* 1991). Furthermore, although 11βHSD2 was detected in human glomerular endothelial cells this isozyme was not detected in endothelium from other human blood vessels (Smith *et al.* 1996).

In addition to the controversy regarding specific cellular localisation of 11βHSD isozymes there is also debate concerning which isozyme is responsible for activity in vascular preparations. Furthermore, the direction of the isozyme *in vivo* has not been clarified. Vascular 11βHSD2 is believed to be a unidirectional enzyme metabolising glucocorticoids to their inactive 11keto-metabolities (Souness *et al.* 2002). In contrast, the directionality of 11β-HSD1 is less clear: for whilst this enzyme is thought to be an exclusive reductase *in vivo* (Kotelevtsev *et al.* 1997; Jamieson *et al.* 1995; Jamieson *et al.* 2000) it exhibits bi-directional activity in VSMC homogenates, cultured VSMCs, and in intact rat aortic rings *in vitro* (Brem *et al.* 1995; Monder & Lakshmi 1989; Brem *et al.* 1998; Souness *et al.* 2002). It has been postulated that the change in directionality between intact cells and homogenates reflects the intracellular localization of 11βHSD1 within the lumen of the endoplasmic reticulum (ER), where neighbouring enzymes may be powerful generators of the reduced co-substrate NADP phosphate (NADPH) (Leckie *et al.* 1998; Seckl & Walker 2001). It is possible that described 'bidirectionality' of 11βHSD1 may reflect cell disruption

through tissue homogenisation or *in vitro* preparations and release of 11βHSD1 in to assay media.

1.2.7 Effect of 11βHSD Inhibition/ Deficiency on Vascular Function

Whilst discrepancies in the literature exist with regard to the cellular localisation of 11βHSD and activity direction *in vitro* and *in vivo* there is general agreement that these isozymes alter vascular function (Brem *et al.* 1998; Hadoke *et al.* 2001; Souness *et al.* 2002). 11βHSDs modulate the influence of glucocorticoids on blood pressure (Walker & Williams 1992) and non-specific inhibitors of 11βHSDs increase the contractile responses to adrenoreceptor agonists and angiotensin II *in vitro* (Walker *et al.* 1994a; Brem *et al.* 1997) and *in vivo* (Teelucksingh *et al.* 1990; Ullian *et al.* 1996).

Initially, the influence of non-specific 11βHSD inhibitors was thought to be as a consequence of inhibiting 11βHSD1 dehydrogenase activity. With the cloning of 11βHSD2 (Albiston *et al.* 1994; Agarwal *et al.* 1994) and appreciation of the almost exclusive reductase activity of 11βHSD1 *in vivo*, the role of 11βHSD2 to influence vascular tone has been acknowledged. More recently with the development of mice deficient in 11βHSD1 or 11βHSD2 the effects of 11βHSD isozymes on the vascular tone has become clearer (Kotelevtsev *et al.* 1997; Kotelevtsev *et al.* 1999). 11βHSD2 deficient mice are hypertensive, have increased vascular contractility to Ang II and norepinephrine and reduced endothelial dependent relaxation with altered nitric oxide metabolism (Hadoke *et al.* 2001; Christy *et al.* 2003). Whilst an *in vitro* paper using rat aorta incubated with 11βHSD1 anti-sense RNA oligomers had suggested

that 11βHSD1 would influence vascular contractile responses (Souness *et al.* 2002) 11βHSD1 deficient mice had no abnormalities of vascular tone *in vitro* or *in vivo* (Hadoke *et al.* 2001).

Whilst the vascular phenotype of 11βHSD1 deficiency is not clear, there are data that suggest vascular function could be influenced by 11βHSD1 in ischaemia and following vessel wall injury. In these patho-physiological situations, the inflammatory cascade that glucocorticoids traditionally modulate (Perretti & Ahluwalia 2000; Barnes 1998), plays an important role and that could be influenced by 11βHSD1 (Cai *et al.* 2001; Longenecker *et al.* 1982; Longenecker *et al.* 1984).

Indeed 11βHSD1 expression and activity was increased in vascular smooth muscle cells in culture in the presence inflammatory cytokines (Cai *et al.* 2001). Perhaps indicating that the vessel wall responds to local vascular injury by regenerating glucocorticoids through 11βHSD1 to modulate any inflammatory reaction.

In addition vessel wall proliferative responses in atherogenesis or following intravascular injury could be influenced by vessel wall 11βHSD1 for glucocorticoids are recognised to decrease vascular smooth muscle cell mitosis in culture (Longenecker *et al.* 1982; Longenecker *et al.* 1984). Such anti-proliferative responses have been suggested to account for the effect of glucocorticoids to diminish restenosis after angioplasty (Berk *et al.* 1991; Lincoff *et al.* 1997; Radke *et al.* 2004; Reimers *et al.* 1998; Versaci *et al.* 2002) but may equally alter other mitotic responses of the vessel wall for example angiogenesis.

The absence of any angiogenic phenotype in 11βHSD1 deficiency is not contrary to these conclusions since it has been observed with other vascular factors that the angiogenic influence of these factors only becomes apparent in the presence of vessel

injury or patho-physiological stimulus (Carmeliet *et al.* 2001; Hodivala-Dilke *et al.* 1999; Bader *et al.* 1998; Carmeliet & Collen 2000; Carmeliet *et al.* 1998). 11βHSD1 may therefore contribute to the vascular control of inflammation and influence processes such as atherosclerosis, restenosis or angiogenesis. Furthermore since angiogenesis involves a series of interactions known to be regulated by glucocorticoids, for example cell proliferation, migration, collagen deposition; and occurs often in the context of inflammation, it is perhaps most likely to be influenced by local regeneration of glucocorticoids by 11βHSD1.

1.3 Angiogenesis

Angiogenesis, the formation of new vessels from existing ones, occurs as a result of a complex series of interactions between the vessel wall, the extracellular matrix and cell signalling cytokines (Figure 1.3).

1.3.1 Formation of the vasculature: angiogenesis and vasculogenesis

Successful embryonic development depends upon the formation of a functional complex network of capillary plexuses and blood vessels through vasculogenesis (Conway *et al.* 2001; Carmeliet 2000). This process describes how endothelial cell precursors (angioblasts) migrate, differentiate and assemble into endothelial cell cords to later become a plexus (Conway *et al.* 2001; Carmeliet 2000). The subsequent growth, expansion and remodelling of this network is described by angiogenesis. Both processes contribute to the development of the embryonic vascular tree.

Adult new vessel formation was traditionally thought to occur solely by angiogenesis but it is now recognised that vasculogenesis (through differentiation of circulating bone marrow or organ derived stem cells) may be possible in the mature animal (Jain 2003). Whether initiated by vasculogenesis or angiogenesis, vessel development in the adult is influenced by common factors in a series of processes involving: *formation, stabilisation, branching, vessel remodelling and specialization.*

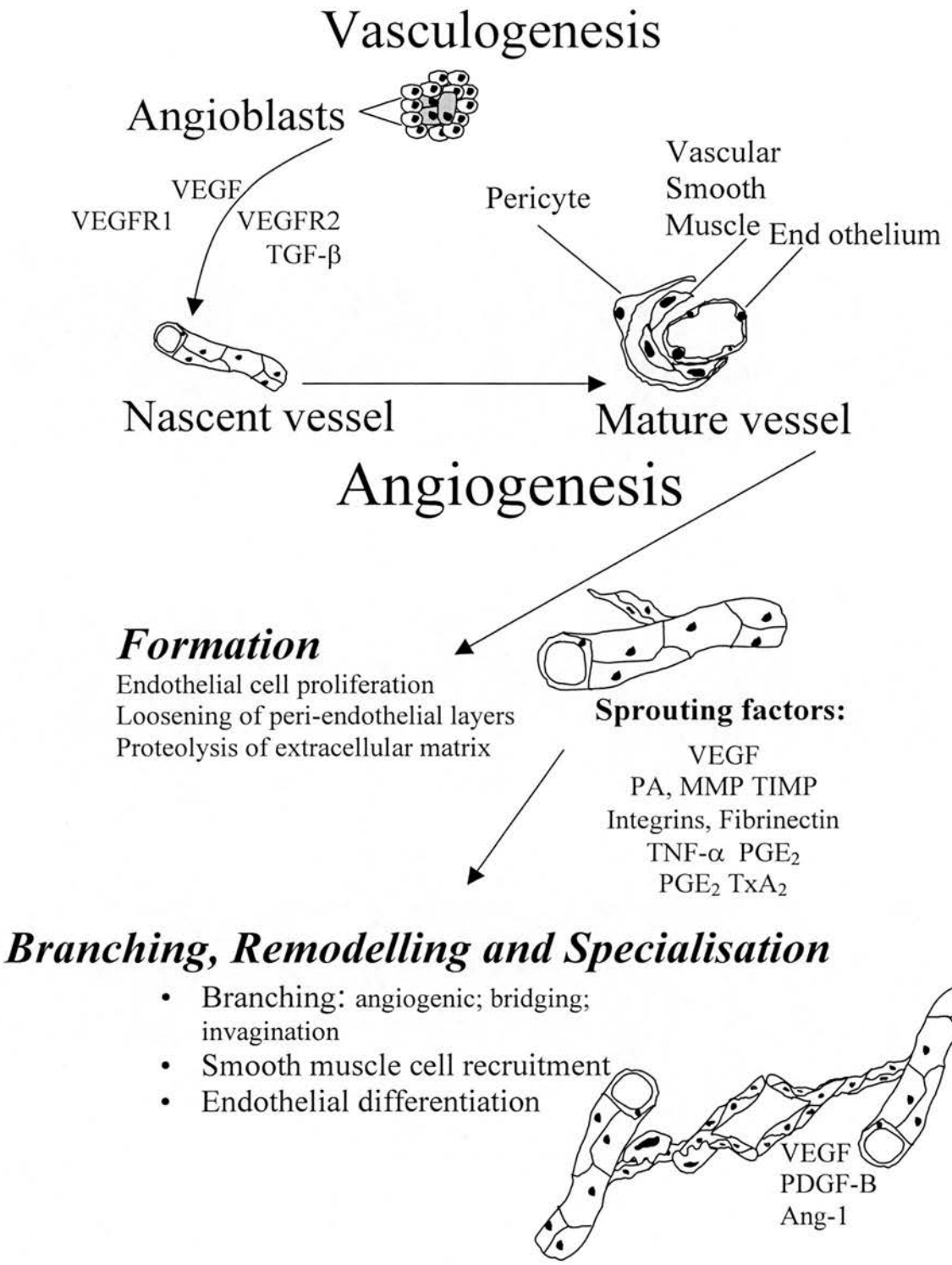


Figure 1.3 Vasculogenesis And Angiogenesis

Vasculogenesis is the formation of new vessels de novo in the embryo. Angiogenesis is the formation of new vessels from the existing vasculature. VEGF vascular endothelial growth factor, VEGFR VEGF receptor, Ang-1 Angiopoeitin 1, PGE_2 prostaglandin E_2, TxA_2 thromboxane A_2, PA plasminogen system, MMP matrix metalloproteinases,TIMP tissue inhibitors of MMP, TGFβ tissue growth factor beta.

1.3.2 Formation of immature vessels

Vasculogenesis- Inception of an embryonic vascular supply requires the formation of a vascular plexus and the production of blood cells. In the embryo, endothelial and haematopoietic cells share a common progenitor: the haemangioblast. In the embryonic yolk sac haemangioblasts differentiate: the inner population become haematopoietic precursors; the perimeter cells become angioblasts. Vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR) 2 and basic fibroblast growth factor (bFGF) influence angioblast differentiation whereas VEGFR1 suppresses haemangioblast commitment (Conway *et al.* 2001; Carmeliet 2000). Vasculogenesis and haemopoiesis are also influenced by Transforming growth factor (TGF)- β1 and TGF-β receptor 2 (Dickson *et al.* 1995). Thus $CD31^{+}$, $CD34^{+}$, VEGFR–2 positive angioblasts migrate extensively and differentiate to assemble primitive plexuses (Jain 2003). Genes identified to influence the fate of haemangioblast include Ets-s, Hex, Vezf1, Hox and GATA family members, basic helix loop helix factors and their inhibitors of differentiation. These genes are also thought to be involved in the angiogenic switch that promotes branching in a mature quiescent vessel (Jain 2003).

1.3.3 New vessel formation

Angiogenic sprouting is facilitated by low tissue oxygen tension that generates hypoxia inducible factors (HIFs), specific transcription factors for many genes whose products are involved in angiogenesis; these include endothelial nitric oxide synthase, VEGF and angiopoietin-2 (Pugh & Ratcliffe 2003). These factors promote the steps associated with initiation of vessel budding. Nitric oxide synthase produces nitric oxide that initiates vasodilation that is an initial phase in the loosening of

vessel wall fabric that allows endothelial cells to migrate (Conway *et al.* 2001; Carmeliet 2000). This process is continued by VEGF release that increases vascular permeability and extravastation of plasma proteins to construct a temporary scaffold (Jain 2003). Angiopoetin-2 (Ang-2) facilitates endothelial cell sprouting by detaching smooth muscle cells and loosening underlying matrix (Conway *et al.* 2001; Carmeliet 2000). In the environment that is established, proliferating endothelial cells may migrate through the vessel wall. Transition of endothelial cells from a quiescent non-mitotic phase of the cell cycle to a state of proliferation is tightly regulated. VEGF, Ang-1, FGF, TNF-α, prostaglandin E_2 and monocyte chemotactic protein (MCP)-1 are recognised positive inducers of endothelial cell proliferation (Conway *et al.* 2001; Carmeliet 2000). Endogenous inhibitors of endothelial cell proliferation include thrombospondin, angiostatin, endostatin, antithrombin III, interferon-β and platelet factor 4 (Conway *et al.* 2001; Carmeliet 2000). Angiogenic stimuli including inflammation and hypoxia modulate the expression of many of these factors and are thus key determinants of endothelial cell fate.

Adaptation of the environment surrounding the vessel wall occurs to accommodate the new vessels. VEGF and other cytokines, including bFGF, tissue necrosis factor α (TNFα), and interleukin-1 (IL1), induce expression of proteolytic proteinases including urokinase and tissue Plasminogen Activators (u-PA and t-PA) and matrix metalloproteinases (MMPs) 1, 3 and 9 (Pepper 2001). Other cytokines limit the angiogenic response by up-regulating the expression of proteolytic inhibitors, for example plasminogen activator inhibitor (PAI)-1 and tissue inhibitors of matrix metalloproteinases (TIMPs) 1 and 2 (Hanemaaijer *et al.* 1993). In this manner a dynamic proteolytic balance in angiogenesis facilitates basement membrane and

matrix dissolution, whilst allowing matrix remodelling and support of vascular buds. The proteolytic balance of the extracellular matrix is one of the factors that determines endothelial cell proliferation and migration (Kraling *et al.* 1999).

Migration of proliferating endothelial cell buds is directed by signalling through integrins $\alpha_V\beta_3$, $\alpha_1\beta_1$, $\alpha_2\beta_1$ $\alpha_5\beta_1$, platelet endothelial cell adhesion molecule (PECAM)-1 thromboxane A_2, ephrins, fibronectin and vitronectin (Conway *et al.* 2001; Jain 2003; Carmeliet 2000). Cell-cell and cell-matrix interactions co-ordinated by these molecules direct endothelial cells to form solid cords that subsequently form a lumen.

1.3.4 Branching, Remodelling and specialisation

Newly formed vessels are stabilised by the recruitment of mural cells and extracellular matrix support. Chemoattractants involved in the procurement of peri-endothelial cells include platelet derived growth factor (PDGF)-B, sphinogosine- 1-phosphate-1 (S1P1), Ang–1 and TGF-β (Conway *et al.* 2001; Carmeliet 2000). Many cells including endothelial and mural cells secrete these factors but it is the presence of specific receptor subtypes on mural cells that determines their proliferation and migration during vascular stabilisation (Jain 2003). The mural smooth muscle cells are derived from different sources; they may differentiate from mesenchymal or endothelial cells or from bone marrow precursors or macrophages (Conway *et al.* 2001; Carmeliet 2000). Coronary vein smooth muscle cells are derived from atrial myocardium whilst epicardial myocardium contributes potential mural cells to the coronary arteries (Conway *et al.* 2001; Carmeliet 2000). Pericytes

are derived from mesenchymal cells that differentiate under the influence of the developing endothelium (Hirschi & D'Amore 1996).

Extracellular matrix support and basement membrane deposition are established through the activities of TIMPs 1 and 2, PAI-1, angiostatin and endostatin with elaboration of collagen, fibrillin and elastin from mural cells (Conway *et al.* 2001; Carmeliet 2000). The inhibition of MMP activity during vessel stabilisation enables scaffold construction and initiates the resolution phase of angiogenesis. The final arrangement of the vascular network will be determined by vessel growth, branching and remodelling.

Vessel branching: Branching may occur by several methods: new branches may sprout in an angiogenic fashion; vessels may split into daughter vessels divided by transendothelial bridges; or vessels may branch via intussusceptions with invagination by a cord of extracellular matrix and partitioning of the mother vessel (Conway *et al.* 2001; Carmeliet 2000). VEGF, Ang-1, acidic FGF and renin are all thought to influence vessel branching (Jain 2003). Extracellular matrix factors including basement membrane proteases and their inhibitors also modulate vessel branching through influencing endothelial cell survival (Jain 2003).

Vessel specialisation: Differentiation of new vessels into arteries and veins followed by organ specific specialization occurs as a result of micro-environmental factors (Conway *et al.* 2001; Carmeliet 2000), interacting with the extracellular matrix and differences in shear stress (Jain 2003). Arteriogenesis involves the recruitment and proliferation of mural smooth muscle cells and the adaptation of the vessel interstitial matrix. Smooth muscle cells in response to bFGF, PDGF-B and TGF-β1 released from activated endothelial cells proliferate and "muscularize" immature vessels.

Interstitial matrix components provide the developing artery with viscoelastic properties (elastin and fibrillin-2) and structural strength (collagen and fibrillin-1) (Pepper 2001). Specializations of cell-cell junctions in the endothelium are determined by host tissue microenvironments. The continuous endothelium of capillaries in the blood brain barrier is successfully established only in the presence of pericytes, astroglial cells expressing glial fibrillary acidic protein and physiological angiotensinogen levels (Lindahl *et al.* 1998). In contrast, VEGF and endocrine gland derived (EG)-VEGF are thought to be essential for the production of discontinuous endothelial cells and fenestrated capillaries of endocrine organs that facilitate hormone synthesis and subsequent release (Jain 2003).

1.3.5 Angiogenesis in pathology

Controlled angiogenesis occurs following pathological insults such as skin laceration or tissue infarction. This type of angiogenesis-in-pathology is initiated by changes in tissue metabolic homeostasis including hypoxia, low pH and abnormal shear stresses (Topper & Gimbrone, Jr. 1999; Semenza 2003). Similar to the processes of embryonic angiogenesis, HIFs, VEGF and NO influence the repairing vascular network (Jain 2003). Following tissue injury there is input from activated platelets that release PDGF and TGF-β (Jain 2003) and also the influence of inflammatory cytokines and matrix proteinases from granulocytes, monocytes and fibroblasts (Tomasek *et al.* 2002). In the initial stages of wound healing, large numbers of immature vessels form. Later some are reabsorbed and the remaining vessels mature (Zawicki *et al.* 1981).

Aberrant, uncontrolled, pathological angiogenesis is observed in primary vascular malignancies and also in tumours of non-vascular origin (Jain 2003). Tumour vessels are distributed in a chaotic manner and do not follow the hierarchical branching pattern of normal vascular networks. The disturbance of vessel spatial organisation is one of the consequences of malignant growth that is not restrained by cell-cell contact or governed by diffusion distances. Vessel wall structure is also abnormal in tumours. Endothelial cells often form an imperfect irregular lining, pericytes have abnormal morphology and mural cells exhibit a patchy distribution (Jain 2003a; Fukumura & Jain 1998; Brown *et al.* 2001). As a result of the abnormal organisation and ultrastructure of tumour vessels the blood flow in tumour vessels is chaotic and the vessels are leaky (Jain 2003b; Hobbs *et al.* 1998; Gazit *et al.* 1997). The tumour micro-environment is thought to be responsible for aberrant angiogenesis. HIFs, VEGF, TGF-β and their receptors are disturbed in malignancy such that restrained dynamic pro and anti-angiogenic processes are uncoupled (Ramanujan *et al.* 2000). The importance of angiogenesis in the progression and metastasis of cancerous growths has led to the development of chemotherapies that target tumour vasculature (Carmeliet & Jain 2000). Identification of specific tumour vessel markers not present on normal vasculature may in the future herald more selective chemotherapies (Huminiecki *et al.* 2002; Sullivan & Bicknell 2003).

1.4 Models of Angiogenesis

The complexity of angiogenesis has given rise to the development of unique experimental assays that study different aspects of angiogenesis. Ideally an assay would be technically straightforward, reliable, reproducible, easy to quantify and physiologically relevant.

In vitro assays of angiogenesis include endothelial cell proliferation, migration, tube formation or explant assays (e.g. aortic ring, endometrium) (Auerbach *et al.* 2003). Generally these are technically straightforward, reliable, reproducible and easy to quantify. Many of these models allow separation of different aspects of angiogenesis and are useful in identifying specific modes of action of putative angiogenesis inhibitors, for example examining the role of test substances to influence VEGF signalling in endothelial cells. However, the physiological relevance of findings from these assays ought to be assessed with consideration to the limitations of the model. For example isolated effects on endothelial cell signalling or tube formation or migration or proliferation *in vitro* may not limit angiogenesis *in vivo* where a combination of the events is required and controlled by cytokines and systemic factors (Staton *et al.* 2004). The explant assays are recognised to come closest to mimicking the *in vivo* situation (Staton *et al.* 2004; Auerbach *et al.* 2003). The aortic ring model (Nicosia *et al.* 1997) assesses endothelial cell proliferation, migration and tube formation; it uses non-proliferating endothelial cells at the time of explantation and includes peri-endothelial cells and supporting matrix. Notwithstanding these benefits, the aortic ring assay does have limitations, angiogenesis is a microvascular event and the use of large explant vessels is not ideal, furthermore by explanting aortic rings from *in vivo* the effects of systemic factors cannot be assessed.

ideal, furthermore by explanting aortic rings from *in vivo* the effects of systemic factors cannot be assessed.

Probably the most widely used *in vivo* angiogenesis assay is the chick chorio-alloantoic membrane (CAM) model (Staton *et al.* 2004). A test substance is implanted onto the CAM through a window cut carefully in the eggshell (Staton *et al.* 2004). It is technically relatively simple and inexpensive. *In vivo* angiogenesis assays in rodents include the corneal angiogenesis assay, Matrigel or sponge subcutaneous implant models and chamber assays. Subcutaneous sponge implantation was chosen in this thesis because it was technically undemanding, reliable, reproducible, humane and did not require lengthy incubation times (Staton *et al.* 2004). The sponge implant model however may cause non-specific immune responses that may themselves lead to an angiogenic reaction (Dellian *et al.* 1996). To avoid non-specific responses and generate physiological angiogenesis reactions *in vivo* pathological models of angiogenesis can be used. For example angiogenesis may be studied in cutaneous wound healing, tumour implantation or in the myocardium following myocardial infarction.

Tumour models of angiogenesis have been used to assess the anti-angiogenic activity of potential chemotherapeutic agents. Tumours can be grown syngenetically (e.g. subcutaneous), orthotopically (in the tissue of origin) or as xenografts in immunodeficient rodents (Staton *et al.* 2004). Quantification of vessel growth is reliable and reproducible and tumour implantation is relatively simple. The patho-physiological relevance of some of these models has been questioned because the tumour microenvironment that determines the chaotic vascular structure in tumours is altered through grafting procedures (Staton *et al.* 2004). In contrast, angiogenesis

in wound healing and following infarction is tightly regulated and establishes an organised, hierarchical, vascular network in the healing tissue. This appears to hold true whether the injury is induced experimentally or not (Singer & Clark 1999).

The number of angiogenesis models available is an indication of the complexity of the process but also the acknowledgement of the necessity to test putative angiogenic substances in a number of environments. In a current review of angiogenesis assays it was recommend, that to understand and interpret the effects of a particular test substance, the use of more than 1 *in vitro* assay using endothelial cells from different sources followed by more than 1 *in vivo* assay is required (Staton *et al.* 2004).

1.5 Modulation of Angiogenesis by Glucocorticoids

During the development of the chick chorioallantoic membrane assay glucocorticoids were discovered to be anti-angiogenic at supraphysiological concentrations (Folkman *et al.* 1983). Further experimental work identified other steroid metabolites of glucocorticoids to be anti-angiogenic and as a group these became known as angiostatic steroids (Folkman & Ingber 1987). The initial investigators identified steroid-induced histological changes in: ***inflammation, cell migration, cell proliferation and extracellular matrix integrity*** (Figure 1.4) (Folkman & Ingber 1987).

Figure 1.4 Proposed Angiostatic Mechanisms of Glucocorticoids

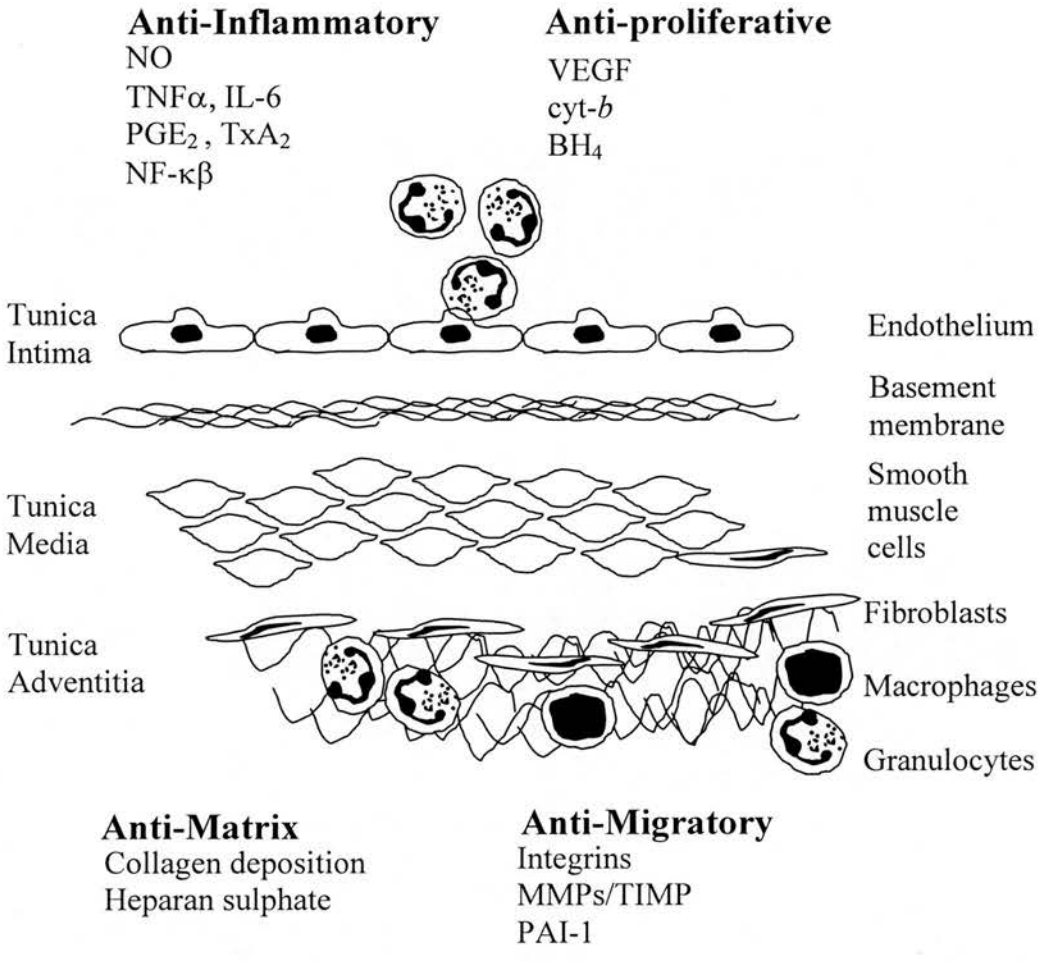


Figure 1.4 Proposed Angiostatic Mechanisms of Glucocorticoids

Anti-inflammatory, anti-proliferative, anti-matrix or anti-migratory activities are postulated mechanisms for the angiostatic effects of glucocorticoids. Interactions between glucocorticoids and key mediators of these responses (which are listed) are reported.

NO nitric oxide, TNFα Tissue necrosis factor alpha, IL-6 interleukin 6, PGE_2 prostaglandinE_2, TXA_2 thromboxane A_2, NF-κβ nuclear factor kappa beta, VEGF vascular endothelial growth factor, BH_4 tetrahydrobiopterin, MMP matrix metalloproteinases, TIMP tissue inhibitors of MMP, PAI-1 plasminogen activator inhibitor 1.

1.5.1 Inhibition of Inflammation

Physiological and pathological angiogenesis are intimately related to inflammation and share many common mediators (Haas *et al.* 2000; Kayisli *et al.* 2002; Walsh 1999; Campochiaro *et al.* 1999; Colville-Nash & Scott 1992). Similar processes govern the initiation of inflammation and angiogenesis; common to both are vasodilation, increased vascular permeability and migration of inflammatory cells (Barnes 1998; Conway *et al.* 2001). Nitric oxide, VEGF, TNF α, IL-6, prostaglandin E_2 (PGE_2) thromboxane A_2 (TxA_2) and transcription factor NFκβ, are pro-angiogenic mediators that are up-regulated by inflammation and suppressed by large concentrations of glucocorticoids (Wallerath *et al.* 1999; Grose *et al.* 2002; Hasan *et al.* 2000; Barnes 1998; Yossuck *et al.* 2001; Beer *et al.* 2000; Blomme *et al.* 2003). Constitutive eNOS and inducible iNOS expression and activity are reduced by glucocorticoids *in vitro* (Whitworth *et al.* 2002) in part by decreasing the availability of L-arginine and tetrahydrobiopterin (BH_4) (Simmons *et al.* 1996; Wallerath *et al.* 1999). Clinical studies in Cushing's syndrome patients suggest that vascular nitric oxide metabolism is similarly impaired *in vivo* in the presence of excess glucocorticoids (Saruta *et al.* 1986). VEGF is an important angiogenic cytokine expressed by vascular smooth muscle cells and other cells in response to cytokines, reduced oxygen tension and cell differentiation (Conway *et al.* 2001). Glucocorticoids inhibit VEGF mRNA expression and stability in different cell culture systems (Gille *et al.* 2001; Wen *et al.* 2003) and physiologically relevant concentrations of cortisone and cortisol (10nM) inhibited VEGF mRNA expression in human vascular smooth muscle cells (Nauck *et al.* 1998). An important concept in studies examining the expression of VEGF is to consider the influence of hypoxia.

Hypoxia is an important angiogenic stimulus through induction of hypoxia-inducible transcription factors (Pugh & Ratcliffe 2003), the modulation of these responses by glucocorticoids is reported but with conflicting results depending on the model, degree of hypoxia, steroid concentration and angiogenic factor assessed (Michalopoulos *et al.* 2003; Fischer *et al.* 2001; Klekamp *et al.* 1997; Yossuck *et al.* 2001). Pro-angiogenic cytokine TNF α expression is also induced by hypoxia and is suppressed by pharmacological concentrations of glucocorticoids *in vivo* in rodent hypoxia-induced retinopathy (Yossuck *et al.* 2001). Glucocorticoid-induced changes in transcription commonly occur via antagonism of the effects of transcription factors AP-1 and NF-κB. Studies to examine the effects of glucocorticoids on these transcription factors in hypoxia have been performed and it would appear that dexamethasone induced AP-1 expression does not regulate VEGF expression in hypoxia (Finkenzeller *et al.* 1995). Nevertheless glucocorticoids at therapeutic concentrations inhibit angiogenesis *in vivo* in hypoxic conditions for example in tumours (Hasan *et al.* 2000; Penhaligon & Camplejohn 1985; Ziche *et al.* 1985; Folkman *et al.* 1983). It is possible that in physiological hypoxic systems other processes like tissue necrosis may inhibit angiogenesis through release of angiogenic inhibitors from the extracellular matrix (Ortega & Werb 2002) (for example endostatin, thrombospondins and tumstatin) and contribute to the effect of angiostatic steroids.

In vivo wound healing models in mice have investigated whether endogenous glucocorticoids are sufficient to tonically repress inflammation (Grose *et al.* 2002). In comparison to wild type controls, in a cutaneous wound healing model the expression of IL-6 expression but not TNFα was increased in transgenic mice that

had defective glucocorticoid receptors which were unable to bind to DNA (Grose *et al.* 2002). This glucocorticoid receptor dependent glucocorticoid effect was associated with an initial increase in wound granulation tissue and mature wounds with normal histological appearance. Other studies have examined the influence of endogenous steroids to regulate inflammation using RU38486 (a glucocorticoid receptor antagonist) and found conflicting results when comparing humans with rodents (Laue *et al.* 1988; Laue *et al.* 1990; Leech *et al.* 2000a). This may reflect differences in the complexity of the immune systems, the dose of drug administered or extent of injury sustained. Attempts have been made to develop *in vivo* angiogenic models to distinguish glucocorticoid angiostatic and anti-inflammatory responses (Hori *et al.* 1996; Kenyon *et al.* 1996). To date however this has not been possible and probably reflects the degree of commonality in the cascades governing both responses. Arachidonic acid metabolites PGE_2 and TxA_2 are good examples of factors shared by both processes and whose production is suppressed by glucocorticoids *in vitro* and *in vivo* through phospholipase A_2 and cyclo-oxygenase-2 inhibition (Blomme *et al.* 2003; Leahy *et al.* 2000; Herschman 1994; Masferrer & Seibert 1994).

The anti-inflammatory effects of glucocorticoids although initially employed to improve the efficiency of an *in vivo* angiogenesis model appear to influence the processes of angiogenesis (Folkman *et al.* 1983; Hori *et al.* 1996). The importance of the anti-inflammatory effects to the overall angiostatic effects of glucocorticoids is difficult to assess but in light of the extent of inflammation in processes involving angiogenesis such as wound healing, tumour growth and also endometrial cycling and muscle hypertrophy it is likely that this is an influential component.

1.5.2 Inhibition of Cellular Migration

Angiogenesis requires the movement of endothelial and peri-endothelial cells through the vessel wall and into the extracellular matrix (Conway *et al.* 2001; Carmeliet 2000). Inflammatory and pro-angiogenic cytokines such as IL6 and VEGF promote changes in vascular structure, cellular activity and extracellular matrix integrity to facilitate new vessel sprouting (Conway *et al.* 2001; Perretti & Ahluwalia 2000; Carmeliet 2000). Chemokine concentration gradients, cell surface integrins, adhesion molecules and matrix proteolysis determine the direction of vessel growth and cell migration (Sottile 2004; Perretti & Ahluwalia 2000). This interplay between cell adhesion, migration and matrix proteolysis has been targeted by pharmaceutical companies for anti-angiogenic therapies and involves processes regulated by glucocorticoids (Pitzalis *et al.* 2002; Preissner *et al.* 1997; Polat *et al.* 2002).

Glucocorticoids influence cell migration through anti-inflammatory effects and by direct effects on integrins and adhesion molecules (Pitzalis *et al.* 2002; Wang *et al.* 2002; Schoeler *et al.* 2003). Integrins provide a physical connection for endothelial and other cells to extracellular matrix proteins and some have been identified as pro-angiogenic whereas others appear to inhibit new vessel formation (Preissner *et al.* 1997; Hynes 2002). Integrin expression is increased in an inflammatory environment (Pitzalis *et al.* 2002; Schoeler *et al.* 2003) as occurs in angiogenesis and glucocorticoids would be expected to antagonise such induction. Glucocorticoids are also associated with altered integrin expression likely to produce an angiostatic influence for example pro-angiogenic integrin $\alpha_I\beta1$ and subunits α_I, α_{II}, and $\beta1$, expression was suppressed in several different cell lines (Zoppi *et al.* 1998; Zhang *et al.* 1993; Ryu *et al.* 1999; Gronowicz & McCarthy 1995; Gohel *et al.* 1995; Brandt

& Vanaman 2000) whereas in others increased expression of anti-angiogenic integrins $\alpha_V\beta5$ and $\alpha_V\beta3$ was observed (Cheng *et al.* 2000). Significantly much of this data was recorded using physiologically relevant concentrations of glucocorticoids, for example 100nM cortisol reduced human placental cytotrophoblast α_{II} integrin subunit expression (Ryu *et al.* 1999).

The environment that new vessels project into is also an important determinant of cell migration and angiogenesis (Sottile 2004; Pepper 2001; Bellon *et al.* 2004). Key modulators of the extracellular proteoglycan milieu are matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) (Sottile 2004; Pepper 2001; Bellon *et al.* 2004). Disturbances of the dynamic balance between MMPs and TIMPs are associated with impaired new vessel formation: angiogenesis in implanted tumours and post myocardial revascularisation was severely impaired in urokinase plasminogen activator (uPA) homozygous deficient mice (Heymans *et al.* 1999; Soff *et al.* 1995). Glucocorticoids are recognised to alter MMP and TIMP expression and synthesis (Pitzalis *et al.* 2002; Wang *et al.* 2002; Perretti & Ahluwalia 2000) and angiostatic steroids are associated with suppression of plasminogen activator activity via increased production of plasminogen activator inhibitor-1 (PAI-1) in bovine aortic endothelial cells (BAECs) (Blei *et al.* 1993). Similar increases in PAI-1 have been observed in human umbilical vein endothelial cells (HUVECs) incubated with glucocorticoids (Zoellner *et al.* 1993) and clinically chronic glucocorticoid therapy is associated with increased plasma concentrations of PAI-1 (Patrassi *et al.* 1997). Other MMP/TIMP systems influenced by glucocorticoids include MMP-2 and its inhibitor TIMP-2 (Pross *et al.* 2002; Wang *et al.* 2002).

Bovine choroidal endothelial cell exposed to large concentrations of trimacinolone exhibited reduced migration, reduced endothelial tube formation and reduced expression of MMP-2 (Wang *et al.* 2002). Primary cultured rat aortic smooth muscle cells *in vitro* similarly had reduced migration in the presence of dexamethasone (100nM) and this was associated with reduced MMP-2 and increased TIMP-2 expression (Pross *et al.* 2002). Differences may exist between rodents and humans in the regulation of cell migration since in the same study human aortic vascular smooth muscle cells were not influenced by dexamethasone and MMP/TIMP expressions were not altered (Pross *et al.* 2002). Similarly leucocyte migration was influenced by endogenous glucocorticoids in rodents but not in clinical studies (Laue *et al.* 1988; Laue *et al.* 1990; Leech *et al.* 2000a).

From the initial observation of endothelial cells exposed to steroids a description of cellular "rounding-up" with retraction of cytoplasmic extensions and detachment from the basement membrane was recorded (Folkman & Ingber 1987). In the context of current understanding of the role of integrins and extrapolation from consistent findings in inflammatory models but also from cutaneous keratinocytes, dendrocytes, cytotrophoblasts, and tumour cells lines (Bator *et al.* 1998; Ryu *et al.* 1999; Zhang *et al.* 1993), glucocorticoids appear to influence integrin expression and matrix proteases. Thus limitations of cell migration and matrix dissolution are potential factors contributing to the angiostatic effects of glucocorticoids.

1.5.3 Influence on Extracellular Matrix: collagen and heparans

Extracellular matrix is a heterogenous layer of proteins, glycocoproteins and proteoglycans that supports established vessels and provides a skeletal scaffold for

new vessels (Ortega & Werb 2002). Constituents of this layer and some of their proteolytic fragments have emerged as important regulators of angiogenesis for example collagen, fibronectin, laminin, thrombospondins and vitronectin (Sottile 2004). Glucocorticoids have been shown to interfere with the synthesis of some of these components in angiogenesis, wound healing, bone formation and skeletal muscle models (Maragoudakis *et al.* 1989; Gu *et al.* 2001; Profita *et al.* 2004; Hernandez *et al.* 2004; Ahtikoski *et al.* 2004). It is therefore probable that some of the angiostatic effects of glucocorticoids are due to reduced synthesis of extracellular matrix components.

Collagen expression and post-translational modification are inhibited by glucocorticoids (Kucharz 1988). Supra-physiological concentrations of cortisone and other angiostatic steroids reduced collagen synthesis and angiogenesis *in vivo* in the chick chorioallantoic membrane model (Maragoudakis *et al.* 1989; Ingber *et al.* 1986). In this model collagen IV is the major component of the collagenous proteins (Maragoudakis *et al.* 1989). Collagen IV is an important constituent of basement membrane a specialized area of extracellular matrix that supports and maintains existing vessel and provides scaffold for the migration of endothelial cells angiogenesis (Ortega & Werb 2002). In a rodent wound healing study endogenous glucocorticoids did not influence collagen IV but did reduce mRNA levels of another basement membrane component, fibronectin (Grose *et al.* 2002). Similarly pharmacological doses of dexamethasone but not endogenous glucocorticoids reduced collagen I and III expression in rodent cutaneous wounds (Grose *et al.* 2002; Oishi *et al.* 2002).

Proteolysis of extracellular matrix collagens liberates anti-angiogenic fragments for example endostatin is formed by the cleavage of collagen XVIII (Kalluri 2002). Clinical studies examining the levels of endostatin in airway secretions in steroid treated asthmatics or joint fluid from steroid treated rheumatoid arthritis patients suggests that glucocorticoids do not alter the levels of these endogenous inhibitors of angiogenesis (Nagashima *et al.* 2000; Profita *et al.* 2004). Pharmacological doses of glucocorticoids were however observed to induce expression of Thrombospondin –1 another extracellular matrix inhibitor of angiogenesis in human and murine trabecular mesh cells (Flugel-Koch *et al.* 2004).

In the initial description of angiostatic steroids Folkman recognised that these compounds disturbed the integrity of the basement membrane (Folkman *et al.* 1983; Crum *et al.* 1985). This appeared to be an early effect that initiated endothelial cell contraction and detachment from the membrane scaffold. In these experiments Folkman observed a synergistic effect of heparin on angiostasis that was in contrast to the pro-angiogenic effect of heparin alone. Heparin is recognised to bind to many angiogenic factors with high affinity and has been used to isolate VEGF and bFGF (Folkman & Shing 1992). It is suggested that heparin pro-angiogenic effects are mediated by a variety of influences including acting as an angiogenic factor chaperone, or by stabilising growth factors or by preventing degradation of angiogenic factors by binding to them in the extracellular matrix (Folkman & Shing 1992). Whether *in vivo* endogenous heparans, heparin sulphate, dermatan sulphate and related polysaccharides in the extracellular matrix or endothelial glycocalyx serve a similar function or facilitate glucocorticoid angiostatic influence is unclear. In the chick chorioallantoic membrane (CAM) *in vivo* assay used by Folkman,

exogenous heparin was required for the angiostatic effect of glucocorticoids. However, in other *in vivo* assays angiogenesis is inhibited by glucocorticoids in the absence of exogenous heparin (Hori *et al.* 1996; Illanes *et al.* 2002; Penhaligon & Camplejohn 1985; Ziche *et al.* 1985). Variations in the dependency of glucocorticoid angiostasis on exogenous heparin is not solely a reflection of the assay used but may also reflect the type of glucocorticoid (Lemus *et al.* 2001) (betamethasone did not require heparin to inhibit angiogenesis in this in vivo study) or the source of heparin (Folkman *et al.* 1983).

Glucocorticoid effects on the extracellular matrix to disrupt collagen metabolism and other proteoglycan components appear to contribute to glucocorticoid angiostatic influence. The majority of evidence for these effects is from the use of large concentrations of glucocorticoids and the influence of endogenous glucocorticoid to determine matrix regulation of angiogenesis is largely unexplored.

1.5.4 Inhibition of Cellular Proliferation

During angiogenesis endothelial, vascular smooth muscle and other peri-endothelial cells proliferate to form new vessels (Conway *et al.* 2001). Stimuli for the angiogenic switch that determines the evolution of a cell from a quiescent stabilised existence to a mitotic, unstable form are complex (King & Cidlowski 1998). Inflammatory cytokines and hypoxia have been identified, as key inducers of the angiogenic switch (Carmeliet 2000). Glucocorticoids inhibit inflammatory cytokine generation (Almawi *et al.* 2002; Wen *et al.* 2003; Gille *et al.* 2001) but also appear to have direct anti-proliferative effects on endothelial cells (Sakamoto *et al.* 1987; Longenecker *et al.* 1982) and have been used as anti-mitotic agents in myeloid and

lymphoid haematological malignancies and prostate cancer (Gaynon & Lustig 1995; Wielckens *et al.* 1987; Fakih *et al.* 2002). Similarly glucocorticoids have been used clinically and in animal models of vascular remodelling following intravascular injury to reduce vascular smooth muscle cell proliferation and reduce rates of restenosis (Radke *et al.* 2004; Berk *et al.* 1991). The ability of glucocorticoids to influence endothelial proliferation *in vitro* and *in vivo* and vascular smooth muscle cell mitosis post angioplasty suggests that the angiostatic effect of glucocorticoids may be determined by influences on cell proliferation, apoptosis and cell survival.

Clinically glucocorticoids are used to inhibit angiogenesis in haemangiomas and biopsy data from such patients indicate that glucocorticoids regulate survival and apoptosis mechanisms (Hasan *et al.* 2000; Hasan *et al.* 2003). Glucocorticoid induced increased expression of mitochondrial cyt *b* was associated with capillary regression *in vitro* and lesion resolution clinically (Hasan *et al.* 2000; Hasan *et al.* 2003). These results are concordant with the traditional view of mitochondria as key organelles in the marshalling of pro-survival and pro-apoptotic factors (Frankfurt & Rosen 2004) and consistent with the mechanism of action of glucocorticoid anti-mitotic activities in haematological malignancies (Frankfurt & Rosen 2004). Other data from haemangioma studies suggest that glucocorticoids influence different aspects of apoptosis (Hasan *et al.* 2003). Increased expression of clus/apoJ, a glycoprotein associated with programmed apoptosis was recorded following glucocorticoid therapy and associated with capillary regression (Hasan *et al.* 2003).
In vivo cortisone in supra physiological concentrations inhibited endothelial cell proliferation (Sakamoto *et al.* 1987). A mechanism for repression of endothelial cell

proliferation could be the influence of glucocorticoids on the synthesis of tetrahydrobiopterin. Tetrahydrobiopterin is a co-factor for the production of nitric oxide and has been shown to be important in the proliferation of endothelial cells (Marinos *et al.* 2001). Glucocorticoids reduce the production of tetrahydrobiopterin through inhibiting of the expression of a rate-limiting enzyme, GTP cyclohydrolase (Johns *et al.* 2001).

Glucocorticoid effects on cell proliferation, survival and apoptosis interplay with the many factors contributing to the processes of angiogenesis. Few studies indicate a direct anti-proliferative effect of large concentrations on endothelial cell proliferation (Sakamoto *et al.* 1987) but clinically therapeutic glucocorticoids induce apoptosis in proliferating capillary haemangiomas (Hasan *et al.* 2000; Hasan *et al.* 2003) and reduce restenosis post angioplasty (Versaci *et al.* 2002). Regulation of cell cycling is a genetically controlled event that would be susceptible to glucocorticoid modulation although other factors may provide steroid targets such as co-factor availability (Johns *et al.* 2001)and mitochondrial function (Hasan *et al.* 2000; Hasan *et al.* 2003). The diversity of glucocorticoid action and evidence from clinical and *in vivo* studies suggest that anti-proliferative effects of glucocorticoids would contribute to their angiostatic properties.

In summary- Consistent observational evidence exists for an angiostatic effects of glucocorticoid at supra physiological concentrations. Although there is a vast amount of circumstantial evidence for the effects of glucocorticoids on relevant pathways, few studies have directly examined possible mechanisms mediating angiostasis. This may reflect the complexity of angiogenesis in conjunction with the diversity of glucocorticoid actions. The circumstantial evidence suggests that glucocorticoids influence angiogenesis through anti-inflammatory, anti-migratory, anti-extracellular matrix and anti-proliferative mechanisms. Whether these are all relevant or all equal in importance to angiogenesis *in vivo* requires further investigation. The influence of endogenous glucocorticoids on angiogenesis has not been assessed and is the subject of this thesis. Some of the circumstantial evidence for glucocorticoid effects on inflammation, matrix interactions and anti-proliferative data does rely on physiologically relevant steroid concentrations and may well have relevance to the findings of this thesis.

1.5.5 Modulation of Angiogenesis by 11βHydroxysteroid Dehydrogenases

11βHSD isozymes in the vessel wall may influence the angiostatic effects of glucocorticoids by altering steroid concentrations locally at the site of angiogenesis. 11βHSD1 amplification of glucocorticoid effects could produce an anti-mitotic effect and an anti-prolifertive role has been described for 11βHSD1 in rat osteosarcoma cells (Rabbitt *et al.* 2003). In contrast 11βHSD2 deactivation of glucocorticoids may produce increases in cell proliferation and a pro-mitotic effect has been described for 11βHSD2 in several endocrine cancers (Rabbitt *et al.* 2003).

No abnormalities of vascular development have been detected in transgenic mice deficient in either 11βHSD type 1 or 2 (Kotelevtsev *et al.* 1997; Kotelevtsev *et al.* 1999). Whilst 11βHSD1 deficient mice have no apparent vascular phenotype it is not unusual for deficiencies of angiogenic factors to be revealed only in pathology (Carmeliet & Collen 2000). Angiogenesis in pathology is influenced by inflammation and it is therefore noteworthy that vascular smooth muscle cell 11βHSD1 was recently observed to be up regulated by inflammatory cytokines (Cai *et al.* 2001). Thus 11βHSD1 in the vessel wall is ideally located and regulated to respond to angiogenic stimuli.

1.6 Hypothesis & Aims

It is evident that glucocorticoids have the ability to attenuate the growth of new blood vessels. What is less clear, however, is whether endogenous glucocorticoids have a clinically significant influence on angiogenesis and, furthermore, whether this influence is modulated by the activity of 11βHSD activity within the vascular wall. Given that altered angiogenesis is central to many disease pathologies and, thus, responsible for considerable morbidity and mortality it is obviously desirable to clarify these relationships. Increased knowledge of the roles of glucocorticoids and 11βHSDs in regulation of vascular growth will extend our understanding of the processes leading to disease development and may identify potential targets for therapeutic manipulation of angiogenesis. Consequently, the work described in this thesis addresses the hypotheses that endogenous glucocorticoids induce a glucocorticoid receptor-dependent inhibition of angiogenesis that is modulated by 11βHSD1 activity within the vascular wall.

In order to investigate this hypothesis, the following aims were addressed:

1. To develop methods for assessing the effects of glucocorticoids on angiogenesis *in vitro*, *in vivo* and in disease pathology.
2. To determine whether endogenous glucocorticoids inhibit angiogenesis by stimulation of the glucocorticoid receptor.
3. To determine whether 11βHSD1 activity in the vascular wall amplifies glucocorticoid-mediated inhibition of angiogenesis.

Chapter 2

Materials and Methods

Materials

Unless otherwise stated all chemicals, reagents and drugs were purchased from Sigma, UK. All HPLC grade solvents were purchased from Rathburn Chemicals, UK. All radioactivity was purchased from Amersham, UK. Different sources are otherwise indicated.

2.1 Buffers and Solutions

Phosphate buffered Saline (PBS): 0.1M phosphate buffer with 137mM NaCl, 2.7mM KCl in distilled water, pH 7.4, autoclaved before use.

Krebs'-Ringer Bicarbonate (KRB) Buffer: 118mM NaCl, 3.8mM KCl, 1.19mM KH_2PO_4, 2.54mM $CaCl_2$, 1.19mM $MgSO_4$, 25mM $NaHCO_3$ in distilled water, pH 7.4. Stored at 4°C and supplemented with 0.2% glucose immediately before use.

Borate Buffer: 0.133M Boric acid, 67.5mM NaOH + HCL, pH7.4 containing 0.5% bovine serum albumin, 1% methanol and 0.1% ethylene glycol.

Tris-Buffered Saline (TBS): 0.05M Tris-HCl, 0.15M NaCl pH 7.6

Buffer C: 300mM NaCl, 1mM EDTA, 50mM Tris pH 7.7, containing 10% glycerol

Modified Buffer C: 50mM Na acetate, 1mM EDTA, 50mM Tris pH 6, containing 10% glycerol

2.2 Drugs and Steroids used in *in-vitro* studies

Carbenoxolone: 61.47mg carbenoxolone was made up to 10ml with ethanol (10^{-2}M solution), then 10µl was added to 10ml deionised water (10^{-5}M solution). Stored at -20°C in 1ml eppendorf tubes.

Cortisol: 36.25mg cortisol was made up to 10ml with ethanol (10^{-2}M solution), then 10µl was added to 10ml deionised water (10^{-5}M solution). Stored at -20°C in 1ml eppendorf tubes.

Corticosterone: 34.65mg corticosterone was made up to 10ml with ethanol (10^{-2}M solution), then 10µl was added to 10ml deionised water (10^{-5}M solution). Stored at -20°C in 1ml eppendorf tubes.

11 Dehydrocorticosterone: 34.44mg 11dehydrocorticosterone was made up to 10ml with ethanol (10^{-2}M solution), then 10µl was added to 10ml deionised water (10^{-5}M solution). Stored at -20°C in 1ml eppendorf tubes.

Indomethacin: 35.78mg dissolved in 30ml distilled water (final concentration 10^{-2}M). 1ml aliquots stored at -20°C in 1ml eppendorf tubes.

Nω-Nitro-$_L$-Arginine (L-NNA): 21.92mg dissolved in 30ml distilled water (final concentration 10^{-2}M). 1ml aliquots stored at -20°C in 1ml eppendorf tubes.

RU38486: 42.96mg RU38486 was made up to 10ml with ethanol (10^{-2}M solution), then 10µl was added to 10ml deionised water (10^{-5}M solution). Stored at -20°C in 1ml eppendorf tubes.

Spironolactone: 41.66mg spironolactone was made up to 10ml with ethanol (10^{-2}M solution), then 10µl was added to 10ml deionised water (10^{-5}M solution). Stored at -20°C in 1ml eppendorf tubes.

Vascular Endothelial Growth Factor (VEGF): Human recombinant Vascular Endothelial Growth Factor $_{A165}$ (Sigma, UK) was reconstituted according to manufacturers instruction: 10µg added to 10ml phosphate buffered saline containing 0.1% bovine serum albumin 1ml aliquots stored at -20°C in 1ml eppendorf tubes.

Fura-2AM: 100µg of Fura-2AM (Calbiochem, UK) was dissolved in 100µl of dimethyl sulphur oxide (DMSO) (Sigma, UK).

2.3 Animals

Male C57Black 6 mice were obtained from Charles River, UK at 10-12 weeks of age. Homozygous null male 11βHSD1 (bred to be congenic) were bred in-house at the MFAA, University of Edinburgh, or the Biomedical Research Facility, Western General Hospital, Edinburgh, UK.

Animals were maintained under controlled conditions of light and temperature (21-22°C), and allowed free access to standard chow (Special Diet Services, UK) and drinking water.

2.4 Angiogenesis *in vitro*: Mouse Aortic Ring Assay

The aortic ring *in vitro* model of angiogenesis was chosen because it accurately represents the major processes of angiogenesis: endothelial cell proliferation, migration and branching capillary network formation (Auerbach *et al.* 2003; Staton *et al.* 2004). Initial descriptions of this model using rat tissue included electron microscopy data describing the formation of luminised capillary tubes consisting of endothelial and peri-endothelial cells (Nicosia & Ottinetti 1990).

2.4.1 Aortae Collection

Mice 10-12 weeks old were killed humanely and under sterile conditions the thoracic aorta was removed, washed in serum free MCDB 131 medium (Invitrogen, UK) at room temperature, cleaned of periadventitial tissue and divided into 1-3 mm rings (time from death to tissue embedding in Matrigel approximately 30min).

2.4.2 Culture conditions

Aortic rings were embedded in Matrigel (Becton Dickinson, UK) in 24 well plates (VWR International, UK) and incubated at 37°C for 7-14 days. Matrigel was prepared by liquefying overnight on iced water at 4°C; 100µl was used as basal layer in each well of a 24 well plate and allowed to gel by incubating at 37°C for 20mins.

1 aortic ring was placed in each well, covered with 200 µl of Matrigel and incubated at 37°C for 20mins. Once the Matrigel had gelled the embedded aortae were cultured in 1ml of serum free MCDB 131, containing heparin, ascorbic acid, GA1000 (supplied as aliquots product number CC3162, Cambrex Biosciences, UK) and vehicle or treatment compound. Media were changed every 48hours. For each set of conditions 3 wells were used thus experiments were performed in triplicate.

2.4.3 Quantification of Angiogenesis

Aortic rings were viewed in the 24 well plates using a Karl Zeiss Axioskop inverted light microscope at x 50 magnification. Image analysis was performed using image analysis software [The Microcomputer Imaging Device (MCID) (Imaging Research Inc, Canada)] and a Pentax 250 live-feed camera.

Measurements were taken of aortic ring circumference, diameter and area. The number of vessels was counted daily and computer image analysis calculations of total area occupied by new vessels, and the maximum and average diameters of the total area occupied by new vessels were recorded.

2.4.4 Vessel characterization

The endothelial nature of the new vessel was confirmed using fluorescent labelled acetylated low-density lipoprotein (DiI-Ac-LDL) (Biogenesis, UK) (Rohan *et al.* 2000). On day 7 culture medium was changed, 50µl of DiI-Ac-LDL stock (200µg/ml) added to each well and incubated in standard conditions for 48 hours. Wells were washed with probe free media and rings examined using Hamamatsu Orca ER CCD camera with Chroma 83000 filter set (with Texas Red excitation).

Images were captured using in house scripts in iPLab spectrum (Scanalytics Ltd, UK).

2.5 11βHSD1 activity in intact aortic rings *in vitro*

The ability of 11βHSD1 to act as a reductase or a dehydrogenase has made it difficult to distinguish the functional directionality of the isozyme in vessels. Usually in tissue homogenate activity assays the particular 11βHSD isozyme responsible for dehydrogenase activity can be distinguished by co-factors (NADP or NAD), however in mice co-factor specificity is less exclusive. Thus to determine11βHSD1 functional directionality in vessels activity assays were performed using intact aortic rings.

Aortic rings from 3-6 wild type mice were incubated (24 hours; 37°C) in 1ml of DMEM-F12 medium (Invitrogen, UK) containing ^{3}H-steroid (as below) supplemented with fetal bovine serum (1%), streptomycin (100μg/ml), penicillin (100units/ml) and amphotericin (0.25μg/ml) (Souness *et al.* 2002). 11β-Reductase activity was determined by adding 10pmol [$^{3}H_4$]-11-dehydrocorticosterone (synthesised in house from 1,2,6,7-[$^{3}H_4$]-corticosterone (Amersham Biosciences, UK) using rat placental homogenate (Brown *et al.* 1993). Mouse liver and medium alone were used as positive and negative controls, respectively. 11β-Dehydrogenase activity was determined by adding 10pmol 1,2,6,7-[$^{3}H_4$]-corticosterone. Mouse kidney slices and medium alone served as positive and negative controls. Experiments were performed in duplicate.

After incubation, steroids were extracted from media using C_{18} Sep-pak columns (Waters Millipore, UK). Aortic rings, which contain 2-3% of the added radioactivity were not included in the extraction (Souness *et al.* 2002). [$^3H_4$]-Corticosterone and [$^3H_4$]-11-dehydrocorticosterone were separated by HPLC and quantified by on-line liquid scintillation counting (Walker *et al.* 1991). Enzyme activity was expressed as conversion after subtraction of apparent conversion in negative control wells.

2.6 Intracellular Calcium Responses to VEGF

Pilot studies were conducted to investigate the influence of glucocorticoids on VEGF stimulated calcium transient in human umbilical vein endothelial cells in order to explore the direct angiostatic effects of glucocorticoids.

2.6.1 Human Umbilical Vein Endothelial cell culture

Miss Katherine Shaw cultured HUVECS (Cell Systems, UK) until passage 3 in T-25ml flasks (Clonetics, UK).

At confluence cells were soaked in 5ml HBSS (Clonetics, UK) and separated from the flask by incubating with 5ml trypsin/EDTA at 37°C 10 minutes (Reagent Pack subculture Reagent Kit Cambrex, UK). Trypsin/EDTA was removed and neutralised by adding 5ml of trypsin neutralising solution (TNS) to the flask. The cells were decanted into a 10ml sterile Falcon tube (BD Biosciences, UK) centrifuged at 1000rpm for 5minutes at 18°C, the supernatant drained and the pellet re-suspended in 1ml of MCDB131 medium. A 10µl aliquot of cell suspension was added to 190µl

of 0.4% trypan blue solution. 10μl were placed into a haemocytometer and the number of cells counted. Cells were diluted to give a final concentration of 0.5 million cells/ml. 1ml of cell suspension was subcultured onto a fibronectin (20 micrograms/ ml) coated glass coverslip in a 6 well plate and incubated until confluent. Once confluent (7 days), cells were incubated in serum free MCDB131 supplemented with heparin, GA 1000 and ascorbic acid (EGM-2 Bullet Kit Cambrex Biosciences, UK) overnight. In the morning, cells were treated with vehicle or cortisol (600nM or 16μM) and incubated in serum free conditions, for a further 6 hours. Cells were washed with Krebs-Ringer solution with cortisol (600nM or 16μM) or vehicle and loaded with Fura-2AM (5μM) (Calbiochem, UK) for 20 minutes. Cells were washed with and then bathed in Kreb's-Ringer bicarbonate solution in the presence or absence of cortisol (600nM or 16μM). The fura-2AM loaded coverslips were mounted on a coverslip case and placed in a temperature controlled cell perfusion chamber (Intral Cel UK) on the stage of an (Nikon Diaphot) inverted fluorescence microscope.

2.6.2 Intracellular calcium measurement

Fura-2 fluorescence was excited at 340 and 380 nm and fluorescence emission measured at 509nm using a spectrophotometer with a data acquisition rate of 0.125 Hz. The ratio of intensities obtained from 340/380 excitation pairs was used as measure of $[Ca^{2+}]i$ (Grynkiewicz *et al.* 1985). For each experiment baseline fluorescence was established followed by the addition of 10ng/ml (Faehling *et al.* 2002) human recombinant Vascular Endothelial Growth Factor $_{A165}$ (Sigma, UK). Acquisition of data was continued for 8 minutes.

Calibration of the $[Ca^{2+}]i$ was performed by monitoring the change in fluorescence at minimal and maximal $[Ca^{2+}]i$ responses by adding 5mM sodium-EDTA in the presence of 1.3mM Ca^{2+} followed by the addition of 10mM Ca^{2+} in Kreb's Ringer bicarbonate solution and 20μM ionomycin. The Grynkiewicz equation (Grynkiewicz *et al.* 1985) was then used to calculate $[Ca^{2+}]i$:

Ca2+ (nm) =Kd. β [(R-Rmin)/(Rmax-R)

Kd is the dissociation constant for FURA-2 this is 224 nm

R is the ratio of flouresence (f340/f380)

Rmin is the ratio of fluorescence when the dye is completely free of Ca2+

Rmax is the ratio of fluorescence when the dye is completely saturated with Ca2+

β is the ratio of fluorescence of saturated to free fluorescence at 380nm (fSat/fFree)

Mean Rmin, Rmax and β for control cells was: 0.3351, 1.4503 and 2.75 respectively

Mean Rmin, Rmax and β for cortisol treated wells was: 0.3749, 1.3597 and 2.82. The mean of the β values was used in further calculations.

2.7 Angiogenesis *in vivo*: subcutaneous sponge implantation assay

To validate the *in vitro* findings, which had been observed in the absence of systemic factors such as inflammation, the influence of endogenous glucocorticoids and 11βHSD1 were investigated *in vivo*.

2.7.1 Drugs and Steroids used in polyurethane sponge implantation studies

Corticosterone, cortisol, cortisone, RU38486 or spironolactone were mixed with Silastic elastomer (Silastic 20 medical grade, Dow Corning Corporation, USA) to produce pellets which have been shown to release their steroid at a constant rate for 4 weeks (Cleasby *et al.* 2003). Polyurethane sponge grade XE1700V was obtained as kind gift from Caligen Foam Ltd., UK

Corticosterone: 550mg of corticosterone were added to 1g Silastic elastomer mixed and centrifuged at 13000rpm for 10 seconds. 100mg of curing agent was added, mixed and centrifuged at 13000rpm for 10seconds. The mixture was drawn into a 1ml syringe and allowed to cure overnight at 25°C. Once cured the steroid-impregnated elastomer was removed from the syringe and divided into 50mg pellets.

Cortisol: 275mg of cortisol were added to 1g Silastic elastomer mixed and centrifuged at 13000rpm for 10 seconds. 100mg of curing agent was added, mixed and centrifuged at 13000rpm for 10seconds. The mixture was drawn into a 1ml syringe, injected into capillary tubing (1.22mm external diameter: 0.76mm internal diameter) (Portex Ltd, UK) and allowed to cure overnight at 25°C. Once cured the steroid- impregnated elastomer pellet was removed from the tubing divided into 10-12mg pellets (containing 2.5-3mg of cortisol) and inserted into 1cm x 0.5 cm polyurethane sponge cylinders. Pellets were inserted using a 200µl micropipette tip to pierce the sponge and guide the pellets into the centre of the cylinder.

Cortisone: 300mg of cortisone were added to 1.10g Silastic elastomer mixed and centrifuged at 13000rpm for 10 seconds. 110mg of curing agent was added, mixed and centrifuged at 13000rpm for 10 seconds. The remainder of the procedure was performed as for cortisol pellets. 10-12 mg pellets containing 2.5-3mg of cortisone were used.

RU38486: 166mg of RU38486 were added to 300mg Silastic elastomer mixed and centrifuged at 13000rpm for 10 seconds. 33mg of curing agent was added, mixed and centrifuged at 13000rpm for 10 seconds. The remainder of the procedure was performed as for cortisol pellets. 10-12 mg pellets containing 5-6mg of RU38486 were used.

Spironolactone: 166mg of spironolactone were added to 300mg Silastic elastomer mixed and centrifuged at 13000rpm for 15 seconds. 33mg of curing agent was added, mixed and centrifuged at 13000rpm for 15 seconds. The remainder of the procedure was performed as for cortisol pellets. 10-12 mg pellets containing 5-6mg of spironolactone were used.

Placebo: 1.10g of Silastic gel was added to 110mg of curing agent, mixed, centrifuged at 13000rpm for 10 seconds, drawn into a 1ml syringe, either injected into capillary tubing or left in the syringe, and cured at 25 °C overnight. Post curing procedure was performed as for corticosterone or cortisol pellets as appropriate.

2.7.2 Adrenalectomy

The influence of endogenous glucocorticoids was examined by implanting placebo-impregnated sponges into mice that had undergone adrenalectomy or sham operation. Dr Patrick Hadoke kindly performed the surgery for these animals. Adrenalectomy was performed as described (Livingstone *et al.* 2000b) and during this procedure animals had bilateral placebo impregnated sponges implanted using the incision created for bilateral adrenalectomy. Following surgery sham and adrenalectomy animals were maintained on 0.9% saline (Livingstone *et al.* 2000b).

2.7.3 Subcutaneous sponge implantation

C57Bl6 and congenic 11βHSD1 male mice 8-10 weeks old were anaesthetized with halothane. A 1cm longitudinal incision was made on the lower back and a subcutaneous tunnel was formed, using blunt forceps, from the incision site to each flank. Through the tunnels a sterilized polyurethane sponge (1cmx 0.5cm) cylinder was inserted subcutaneously to each flank. Every animal received 2 sponges.

Intervention agents (vehicle, cortisol, corticosterone, cortisone, RU38486, spironolactone) were administered to the sponges as Silastic implants in 2 different ways. In preliminary experiments implants were placed adjacent to the sponges, in latter experiments Silastic implants were inserted into the sponge. Animals received a placebo-impregnated sponge on one flank as control and a steroid impregnated sponge on the opposite flank.

The skin wound was closed with clips and 0.05ml/Kg subcutaneous (s.c.) buprenorphine analgesia was administered subcutaneously. Mice recovered on a

heating pad at 30°C until moving freely. Normal chow and water was supplied *ad lib*, animals were weighed and had their water intake monitored daily.

Twenty days following sponge implantation mice were euthanised by decapitation; trunk blood was collected in lithium heparin and placed on ice. Thymus, adrenal glands and spleen were removed and weighed. The sponges were removed and bisected for histology or quantification of protein and steroid content.

2.7.4 Quantification of angiogenesis in polyurethane sponges

Half of the sponge for histological examination was placed immediately into 10% formalin for 1 hour, transferred to 75% alcohol for 30 minutes and stored in 90% alcohol prior to paraffin embedding. Paraffin wax processing was performed using program 1 of Tissue Tek Vacuum In filtration Processor (VIP).

Paraffin wax embedded samples were stored at room temperature prior to sectioning. 7μm sections were cut using a microtome (Ernest Leitz 1512) floated in a water bath at 30°C and mounted onto positive electrostatically charged frosted glass slides (BDH Laboratory slides, VWR International, UK). The slides were dried at room temperature placed in an oven at 37°C for 24 hours and stained with haematoxylin and eosin.

Vessels were readily identified by their characteristic shape and from the presence of erythrocytes within their lumens. Two cross sections from each sponge were examined using low power (x100) and Chalkley counting at high power (x250) (Fox *et al.* 1995). Chalkley counting is performed using a 25-point Chalkley eyepiece reticule (Graticules Ltd, Kent UK). At low power the 3 most vascular areas were identified and analyzed using the graticule at 250x magnification. The graticule was

rotated until the highest number of Chalkley reticule points were aligned with vessels and a score out of 25 was achieved. From each section three Chalkley scores were recorded thus a total of six Chalkley scores were obtained for each sponge and the mean of these was used as the Chalkley count.

2.7.5 Polyurethane sponge protein and steroid quantification

The half of the sponge for protein and steroid quantification was placed in ice cold PBS, the Silastic insert was removed and the sponge was homogenized in 2ml ice-cold PBS for 5mins using an Ystral mechanical homogeniser (Scientific Instruments Centre, UK). The homogenate was centrifuged at 2000rpm 4°C for 30 minutes. 40µl of the supernatant was processed for protein content using the Bradford assay (Bradford 1976). A further 750µl of the supernatant was removed, added to7.5ml of ethyl acetate, vortexed and allowed to settle for 5mins. 7ml of the supernatant was removed and dried at 60°C. Samples were then stored at -20°C until steroid radioimmunoassay was performed.

2.7.6 Sponge Protein determination: the Bradford assay

The protein concentration of tissue homogenates was determined colorimetrically by the Bradford assay (Bradford 1976) using a Bio-Rad protein assay kit (Bio-Rad, UK). A range of protein standards (0.1–1.2mg/ ml) was prepared in duplicate in distilled water from the provided protein standard (bovine serum albumin). Protein assay dye reagent (Coomassie Blue G-250) was diluted 1:4 in distilled water and filtered through Whatman No. 1 filter paper prior to use. Diluted protein assay dye reagent (1.96 ml) was added to 40µl of protein standard or appropriately diluted

tissue homogenate in a borosilicate tube, vortexed and left at room temperature for 15 min–1 hour to allow colour development. Absorbance of samples at λ=595nm was measured using a Shimadzu UV/ visible recording spectrophotometer and the concentration of protein in each sample was estimated from the standard curve.

2.7.7 Steroid quantification: Radio-Immuno assay

Plasma preparation: Trunk blood collected onto ice in a lithium heparin containing tube was centrifuged at 3000rpm for 10mins at 4°C the supernatant was removed and stored at -80°C. Stored plasma samples were defrosted and diluted ten fold with borate buffer and heated at 80°C for 30 minutes to denature corticosterone-binding globulin.

Polyurethane Sponge extract *preparation:* Dried polyurethane sponge steroid extracts were re-suspended in borate buffer and x10 and x100 serial dilutions made.

***Steroid standards preparation*:** Depending on the steroid being assayed corticosterone, cortisol or cortisone standards were made with serial dilution.

In a 96 well Falcon Microtest flexible assay plate 20µl of standard or diluted plasma or diluted steroid extract were incubated with 25µl primary antibody (diluted 1:10,000) [polyclonal rabbit anti-corticosterone or polyclonal sheep anti-cortisol or polyclonal rabbit anti-cortisone, 25µl ^{3}H steroid [^{3}H corticosterone (1.5nM), ^{3}H cortisol (1.5nM), ^{3}H cortisone (1.5nM)] and 25µl anti-primary antibody scintillation proximity assay reagent (Amersham). Sample and standards were incubated at room

temperature for 10-15 hours and counted in an LKB Wallac Microbeta counter. A standard curve was constructed and concentrations from sponge extracts and plasma calculated. The lower limit of accurate detection for this assay was 50 nmol/l (Cleasby *et al.* 2003).

2.7.8 High pressure liquid Chromatography

The high-pressure liquid chromatography (HPLC) system comprises an auto-sampler and mobile phase pump (Waters, UK), a symmetry shield RP_{18} 5μm column (Waters, UK) and a radioactivity monitor linked to a scintillation fluid pump (Berthold, UK). The system is controlled by the Winflow computer programme (JMBS Developments, France).

Anti-cortisone antibodies in the radio-immuno assay used to detect cortisone cross-react with cortisol therefore the sponge extract from sponges in animals that had received cortisone were passed through the HPLC column. To separate cortisone from cortisol, steroid extracts from the sponges were re-suspended in 1ml mobile phase (60% water, 15% acetonitrile, 25% methanol). A 180μl aliquot of each sample was injected into the HPLC system. The flow rate of the mobile phase was 1ml/ min. The column temperature was 35°C to maintain stability of retention times. 1M standards of cortisone and cortisone were injected at the start of each study to define the retention times for each steroid. The approximate retention times for cortisol and cortisone were 17-19 min and 14-16 min, respectively (variation reflects slight differences in mobile phase composition and column condition). Mobile phase

containing steroid was collected at the appropriate time points and stored at 4 °C prior to steroid extraction using ethyl acetate.

Following separation of cortisol and cortisone by the column homogenised sponge samples from 11βHSD-1 -/- mice were subjected to ultra violet lamp detection. Ultra violet light absorption can be used to distinguish steroids quantitatively. In this fashion it was possible to determine the presence or absence of cortisone or cortisol in sponge samples.

HPLC with on-line scintillation counting was used to quantify [^{3}H]-corticosterone and [^{3}H]-11-dehydrocorticosterone in culture medium from aortic ring incubations to determine 11βHSD-1 activity.

Steroid extracts were re-suspended in 1ml mobile phase (60% water, 15% acetonitrile, 25% methanol). A 180μl aliquot of each sample was injected into the HPLC system. The flow rate of the mobile phase was 1ml/ min and the flow rate of the scintillant (Quicksafe Flow 2; Zinsser, UK) was 2ml/ min to achieve optimal mixing and counting efficiency. The column temperature was 35°C. Radioactive standards were injected at the start of each batch of samples to determine peak identity. The approximate retention times for [^{3}H]-corticosterone and [^{3}H]-11-dehydrocorticosterone were 7-9min and 10-12min respectively, with greater than 1min between the two peaks. Peaks were less than 1min 30s wide and peak height was at least 50x background.

Following chromatography, the area under each peak was integrated using the Winflow software and used to quantify the percentage conversion of [^{3}H]-corticosterone to [^{3}H]-11-dehydrocorticosterone i.e. 11β-HSD activity. The percentage conversion in each tissue sample was corrected for the "apparent conversion" occurring in tissue blanks included in each experiment, which was always <4%.

2.8 Angiogenesis in Pathology: chronic coronary artery ligation

Experience at mouse chronic coronary ligation and sham procedures was obtained through direct observation, and the performance of non-recovery procedures under the supervision of Dr Isam Sharif Centre of Cardiovascular Sciences University of Edinburgh. A formal assessment of technique was made by Dr Jane Conole University of Edinburgh Veterinary Surgeon.

2.8.1 RU38486

Wild type mice received a subcutaneous 10mg implant (Cleasby *et al.* 2003) containing either vehicle or 5.25mg of RU38486 (manufactured as in 2.7.1) 1 week prior to sham or coronary artery ligation. 7 days following the coronary procedure animals were re-anaesthetised echocardiographied and euthanised. Blood was obtained by intra-cardiac puncture for the measurement of plasma corticosterone and RU38486, hearts and cutaneous wounds were collected and processed for histology.

2.8.2 Mouse Coronary Artery Ligation Surgery

Male C57/Bl6 wild type and 11βHSD1 -/ mice, 12-14 weeks old, were used. Mice were anaesthetised with an intra-peritoneal (i.p.) injection of xylazine (0.018mg/kg), ketamine (100mg/kg), and atropine (600mcg/kg) (Mora *et al.* 2003). Surgery was performed as previously described (Lutgens *et al.* 1999). The thorax was clippered (Wah cordless trimmer, Harvard Apparatus Ltd, Kent UK) and endotracheal intubation was performed using a blunted 12 G needle.

Once intubated, mice were ventilated (HSE-Harvard MiniVent, Harvard Apparatus Ltd, Kent UK) at 120 breaths-a-minute with stroke volume 200µl, breathing oxygen-enriched air.

Buprenorphine (0.05mg/Kg) was administered subcutaneously and eyes were coated with Lacrilube ointment. The shaved skin was cleaned with an antiseptic wipe and under sterile conditions on a heat pad using a microscope at 10x magnification a 1 cm subcutaneous incision was made at the 4^{th} intercostal space anterior axillary line, extending medially. With blunt dissection subcutaneous tissue was separated from fascia covering pectoralis transversus, pectoralis profundus and medial fibres of pectoralis profundus pars abdominalis. The lateral border of pectoralis profundus was lifted with toothed forceps and blunt-dissected from lateral fibres of pectoralis profundus pars abdominalis. A 5.0 mersilk suture was used to tie back the lateral border of pectoralis profundus into a medial position to reveal the medial edge of pectoralis profundus pars abdominalis that was bluntly dissected from fascia of the ribs and intercostal muscles. A 5.0 mersilk suture was used to tie back the medial edge of pectoralis profundus pars abdominalis in a lateral position. In this fashion the deep fascia covering the ribs and intercostal spaces was revealed. The respiratory

movement of the left lung and the beating heart could be observed at this stage, deep to the intercostal muscles. The fourth intercostal space was confirmed by observing the curvature of the medial border of the left lung. Inferior to the 4th intercostal space the medial border sharply turns to meet the lateral border; superiorly to the 4th inter-space the medial border arches laterally. The landmarks were confirmed by locating an extra-thoracic, emerging branch of the internal thoracic artery parasternally at the 5th intercostal space.

An incision was made in the 4th intercostal muscle. Great care was taken to avoid the underlying viscera by lifting the 5th rib and making first a puncture with blunt curved forceps; the forceps were then rotated such that the long axis of their curve was laid parallel to the intercostal space away from the heart. Sharp scissors cut through the intercostal muscle in a lateral direction towards the forceps tip.

The magnification was increased at this stage to 16x. A 1cm tissue clamp was inserted in the 4th intercostal space at the site of the incision. The heart, and medial border of the left lung were visible. The pericardium was carefully lifted as a straight edge by two forceps and delicately moved in a lateral direction. A small hole was made in this sheet near the point of contact with the heart at an area where the pericardium was thin. The sheet was pulled more laterally to rotate the heart on its long axis as required to visualise the origin of the left main descending artery (LMDA). Controlling the illumination and elevating the left atrium using fine smooth tipped forceps assisted identification of the LMDA. The LMDA was typically found appearing beneath the left atrium at the atrial notch coursing from a posterior-lateral direction towards the anterior aspect of the left ventricle; rapid pulsation of bright red blood was seen in its walls.

A 6.0 proline suture was inserted into the myocardium 2-3mm inferior to the intersection of LMDA with the left atrial notch. The suture was passed beneath the artery but not into the ventricular cavity and brought out 1-2mm medial to the insertion site. The suture was tied, the heart rhythm was immediately noted to alter and the ventricular myocardium seen to pale.

The pericardium was replaced, the clamp removed and the intercostal space sutured using 5.0 mersilk with discontinuous sutures. The mersilk ties in the borders of pectoralis profundus and pectoralis profundus pars abdominalis were removed; the muscle borders were teased into their former positions. Negative pressure was created with the chest cavity with gentle digital pressure and this held the muscles in place over the 4^{th} intercostal space.

The skin was closed with 5.0 mersilk with continuous sutures. On completion of surgery animals received intraperitoneal atipamazole (AntiSedan, an α-adreno-receptor antagonist) (5mg/kg). Recovery sufficient for extubation was characterised by self-breathing movements, whisker twitching and myoclonic jerks. Animals were transferred to a recovery cage with heating pad, oxygen (delivered by funnel into which the mouse head was placed) and *ad lib* saline. The oxygen was removed; the cage remained on a heat pad for the next 24 hours. Animals post operative care and analgesia was performed in accordance with Edinburgh University Biological services surgery guidelines.

At days 1, 3, 5, 7, or 14 animals were anaesthetised i.p. with ketamine, xylazine and atropine as before, underwent echocardiography and prior to recovery were euthanised by cervical dislocation. The heart was removed, bisected across the long axis, weighed, placed in 10% formalin for 24 hours and stored in 70% alcohol.

2.8.3 Quantification of Left ventricular remodeling by echocardiography

Mouse echocardiography was performed using a Diasus ultrasound machine (Dynamic Imaging Livingston UK) (Mora *et al.* 2003). A 10-22MHz transducer was applied parasternally to the shaved chest wall and manoeuvred to obtain 2-dimensional (2-D) images in a parasternal long-axis view of the left ventricle. Images were stored on an optical disk and analysed offline using Diasus software (Dynamic Imaging). Left ventricular (LV) parameters were measured at the mid- papillary level on live 2-D images and averaged from 2 cardiac cycles. Ventricular septal thickness was previously noted not to be affected by left descending coronary artery ligation and thus was not assessed in this study (Lutgens *et al.* 1999).Other measurements taken of LV parameters included left ventricular end diastolic diameter (LVEDD), LV end systolic diameter (LVESD), LV end diastolic area (LVEDA), LV end systolic area (LVESA), posterior wall thickness at end diastole (PWD) and posterior wall thickness at end systole (PWS)]. Left ventricular ejection fraction (EF) ([(LVEDA-LVESA)/LVEDA] x 100) and fractional shortening (FS) ([(LVEDD-LVESD)/LVED] x 100) were used as measures of left ventricular function (Mora *et al.* 2003).

2.8.4 Histological quantification of infarct size and angiogenesis

Excised hearts were fixed in 10% formalin, paraffin embedded and sectioned at 8μm and mounted on positively charged BDH Superfrost glass slides (BDH laboratory supplies, supplied by VWR International Lutterworth UK). Sections were stained with haematoxylin and eosin to measure infarct size (Virag & Murry 2003), or Van

Gieson stain to identify the deposition of collagen or Anti-Von Willebrand factor antibody (Dakocytomation, UK) to label endothelial cells and quantify angiogenesis. Vessels were counted (Virag & Murry 2003) at x 400 magnification (Karl Ziess Axioskop) in the 4 most vascular fields, 2 endocardial 2 epicardial, using a $0.0625mm^2$ reticule; the borders of the reticule were within the infarct.

2.8.5 Immunohistochemistry

Mounted sections were deparaffinised in xylene 2x5 minutes taken through 2x5 minutes in 100% ethanol to be rehydrated and rinsed in water for 2 minutes.

Rat anti –mouse anti-CD 31 antibodies (Pharmingen) were initially used to identify endothelial cells. Endogenous peroxidases were quenched by placing deparaffinised, rehydrated slides in 3% H_2O_2 in methanol for 20 min. 1:100 and 1:500 primary antibody concentrations were used with comparisons of incubation times (overnight or 2 hours), and different antigen retrieval methods. Attempted CD31 antigen retrieval included using 5 min microwave boiling in TBS buffer or a digest in trypsin (Sigma) 1 mg/ml in distilled H_2O for 45 min at 37°C or a digest in 20μg/ml proteinase K (Sigma) in TBS for 15 min at 37°C or incubation for 5 minute microwave boiling in antigen retrieval solution (Dakocytomation) in TBS. A secondary antibody from a Vector kit (Vectastain Elite ABC kit, Vector, UK) was used that contained an anti-biotin antibody attached to a horseradish peroxidase moiety. The secondary antibody was applied at maximum recommended concentration (10μg/ml) and incubated at room temperature for 30 minutes. Slides were washed x2 in TBS and 200μl of Diaminobenzidine (DAB) (Vector, UK) was

used as the chromogen substrate to visualise the peroxidase. Slides were then washed, dehydrated with 70 % alcohol for 3 minutes followed by 2x 5 minute soaks in xylene and coverslip mounted using a permanent mounting media.

These methods did not successfully identify CD-31 antigen staining. For this reason anti-von Willebrand antibody was used to identify an alternative endothelial antigen. Antigen retrieval by incubation with trypsin was performed. 50ml of Tris-Buffered saline (TBS, Dakocytomation, UK) was heated to 37°C (medium heat, 850 Watt microwave, 20 seconds) 0.5ml was removed. 1 porcine trypsin tablet (Sigma, UK) was dissolved in 1ml distilled water; 0.5ml was removed and added to the 45ml of TBS at 37°C. Slides were placed into this solution and incubated for 90 minutes at 37°C. Subsequently slides were mounted in a Sequenza platform and rinsed x2 with TBS. 200µl of rabbit anti-human von- Willebrand factor (Dakocytomation, UK) (1:200, diluted in TBS and 1% bovine serum albumin) were added to the coverslip reservoir of each slide and incubated at room temperature for 2 hours (1:400 primary antibody dilution was also tried but proved to be unsuccessful). Slides were washed with TBS x2 and 200µl secondary antibody added to the coverslip reservoir and incubated for 30 minutes; an EnVision (Dakocytomation, UK) kit was used that contained a secondary anti-rabbit antibody attached to alkaline phosphatase moiety. Slides were washed x2 in TBS and removed from the coverslips. 200µl of Fast red solution (Dakocytomation, UK) that reacts with alkaline phosphatase to produce a red stain was applied to the slides and incubated for 2-5minutes. Slides were rinsed in tap water and coverslip mounted using an aqueous medium.

2.8.6 Plasma Analysis

Plasma for corticosterone was quantified by radio immuno assay as described in section 2.8.7. Plasma RU38486 was assayed using liquid chromatography mass spectrometry (LC/MS/MS). 100 uL of alfaxalone (Sigma, UK) at 2μg/ml was added as internal standard to 0.5 mL of plasma. The solution was acidified with 100 μL of 1M hydrochloric acid; extracted once using 2 mL of ethyl acetate, dried down under nitrogen and reconsituted in 1 mL of mobile phase. A set of standards was prepared using analytic standard RU38486 (Sigma, UK) and alfaxalone following the extraction procedure outlined. Dr Natalie Homer performed the LC/MS/MS quantification technique on a Thermoelectron TSQ Quantum Discovery with attached Surveyor HPLC.

Samples were reconstituted in 75μl mobile phase (70:30 methanol: 5 mM ammonium acetate) and 10 μL were injected onto a C18 column (BDS Hypersil, Thermofinnigan, 50 mm, 2.1mm id, 5μm pore size). Analytes were eluted under a gradient of 70% methanol increasing to 90% over 10 minutes at a flow rate of 300 μL/min at 35°C. Ionisation was achieved by positive ion electrospray, using a spray voltage of 3000 V, sheath gas pressure of 60 psi, auxiliary gas pressure of 5 psi, capillary temperature of 300°C, collision pressure of 1.5. MS/MS was performed on the $[M+H]^+$ ion of alfaxalone (333 *m/z*) with tube lens voltage of 136 V and a collision energy of 18 V monitoring for the ion fragment at 297 *m/z*. Single Reaction Monitoring of the $[M+H]^+$ ion of RU34846 (430 *m/z*) gave a dominant product ion at 372 *m/z* at a collision energy of 18V. Utilising this technique the calibration range of the assay was 0.5-30ng RU34846.

2.8.7 11βHSD Activity

An assay developed for use in tissue homogenates was used to assess 11βHSD activity in the heart (Jamieson *et al.* 1995). As 11βHSD1 functions as a reductase and dehydrogenase in tissue homogenates (Sandeep & Walker 2001), and reductase and dehydrogenase 11βHSD are affected by inflammatory cytokines (Cai *et al.* 2001) both reductase and dehydrogenase activities were assessed. Heart tissue from C57Bl6 mice that had undergone coronary artery ligation or sham 7 days previously and stored at -80^{0}C was thawed on ice placed in 600µl of buffer and homogenized until liquid. 10µl of homogenate was taken for protein quantification as described Section 2.4.4.1. Sample protein concentration was used to standardize the amount of sample added to the assay. Sufficient sample was added to Modified buffer C (for reductase) or Buffer C (for dehydrogenase) to produce a working solution containing 0.2mg/ml of protein. A reductase reaction was established by adding 10ml of ^{3}H-11dehydrocorticosterone to 50ml of cofactor NADPH (2nM) and 190µl of sample/Modified buffer C mixture. Conversely a dehydrogenase reaction was established by adding 10ml of ^{3}H-Corticosterone to 50ml of cofactor NAD (2nM) and 190µl of sample/ buffer C mixture.

Samples were incubated in a water bath at 37^{0}C for 2.5 or 5 hours for the reductase reaction and 5 or 24 hours for the dehydrogenase response. The reactions were terminated by placing the samples on ice. Steroids were extracted with ethyl acetate dried, resuspended in ethanol, separated by TLC and estimated by scintillation counting. Enzyme activity in each direction was expressed as the percent conversion to product (Jamieson *et al.* 1995).

2.8.8 Infarct size measurement

The area of left ventricle affected by infarction was determined as a percentage of left ventricular wall area (Lutgens *et al.* 1999) and measured at direct light microscopy; images were captured using Research Systems Photometric camera and analysed using in-house scripts.

2.9 Cutaneous Wound Healing

Thoracotomy wounds from mice undergoing chronic coronary ligation that had been sutured with 5.0 mersilk (Ethicon, Johnson & Johnson, UK) using a 1mm round-bodied needle were used in these studies. At sacrifice 7 days following chronic coronary ligation the thoracotomy cutaneous wounds were removed by wide excision, placed in 10%formalin and processed for histology. Paraffin embedded skin was sectioned (8μm) and stained with haematoxylin and eosin for morphological analysis or with an endothelial cell marker to quantify angiogenesis.

2.10 Statistics

Data are mean ± SEM. Comparisons were made by ANOVA with least squares difference post hoc tests. Inter-assay- and intra-assay coefficients of variation in wild type mice were 17 % (n=32) and 22 % (n=18), respectively for vessel number in aortic rings after 7 days in culture; 12 % (n= 6) and 12 % (n= 6) for vessel density in sponge implants; 7 % (n=6) and 25% (n=6) in day 7 infarcts and 7% (n=4) and 12% (n=4) for day 7 wounds.

Chapter 3

Blood Vessel Wall 11βHSD1 Regulates Glucocorticoid-induced Angiostasis *in vitro*

3.1 Introduction

Despite extensive literature indicating that glucocorticoids inhibit angiogenesis *in vitro,*(Nicosia & Ottinetti 1990) *in vivo* (Hori *et al.* 1996) and clinically (Hasan *et al.* 2000) the mechanism for these effects are not understood. In addition the potential of vessel wall 11βHSDs to modulate the angiostatic effects of glucocorticoids has not been considered.

Until now therapeutic concentrations of glucocorticoids have been used in angiogenesis assays with a view to using steroids as anti–angiogenesis therapy (Folkman *et al.* 1983). Criticisms of this work concern the concentrations or type of glucocorticoid used and that mechanistic *in vitro* studies are qualitative not quantitative (Folkman & Ingber 1987; Illanes *et al.* 2002; Hori *et al.* 1996; Crum *et al.* 1985). No previous studies have examined the effects of endogenous glucocorticoids to modulate angiogenesis despite reports of impaired wound healing in conditions of chronically elevated endogenous glucocorticoids (Gordon *et al.* 1994) and reduced vascularity in glucocorticoid producing adrenal cortex adenomas in comparison to normal adrenal cortex (Bernini *et al.* 2002).

Angiostatic effects of high concentrations of glucocorticoid have been observed in rat aortic ring assays of angiogenesis (Nicosia & Ottinetti 1990). This model is unique as an *in vitro* assay for it contains all the cellular elements of the vessel wall, and provides a model that incorporates cell proliferation, migration, tube formation and tube luminisation (Nicosia & Ottinetti 1990). The model is recognised to be a useful *in vitro* representation of the *in vivo* process because of the representation of all these different facets of angiogenesis (Staton *et al.* 2004; Auerbach *et al.* 2003). New vessel formation in the aortic ring assay is not influenced by systemic

inflammation and therefore presents an opportunity to investigate the angiostatic effects of glucocorticoids without systemic inflammatory involvement. As a mouse model the assay provides an opportunity to investigate the influence of gene deletions on angiogenesis. The effects of 11βHSD1 on angiogenesis can thus be examined using aortae from transgenic 11βHSD1 null mice. Previously it has been difficult to study the vascular effects of 11βHSD1 as there are no available selective inhibitors of this isozyme (Hadoke *et al.* 2001) and the available non-selective inhibitor carbenoxolone is toxic to endothelial cells (Ullian *et al.* 1996).

In vitro assays to examine the molecular mechanisms of glucocorticoid-induced angiostasis have used therapeutic concentrations and generally have been observational studies (Ingber *et al.* 1986; Sakamoto *et al.* 1987; Cariou *et al.* 1988). No study has examined the effect of endogenous concentrations of glucocorticoids on key angiogenesis factors for instance endothelial cells or VEGF. Endothelial cells are central to new vessel formation, are relatively straightforward to culture and exhibit readily measurable intracellular calcium transients in response to VEGF stimulation (Cunningham *et al.* 1999). Inhibition of VEGF provoked calcium responses in endothelial cells impaired cell proliferation and migration (Cunningham *et al.* 1999; McLaughlin & De Vries 2001). Furthermore several endogenous inhibitors of angiogenesis use this mechanism of action (Jiang *et al.* 2001). No previous study has considered the effects of endogenous glucocorticoids on endothelial cell calcium responses to VEGF.

Therefore the aims of this study were to establish 2 assays to investigate the effects of glucocorticoids on angiogenesis. An *in vitro* model of angiogenesis using mouse aortic ring cultures was established to examine the influence of endogenous

glucocorticoids to modulate angiogenesis. In addition this model was used to investigate the effects of vessel wall 11βHSD1 to determine the influence of glucocorticoids on new vessel formation. A further assay was developed to begin to explore the mechanisms of glucocorticoid and 11βHSD1 influence on angiogenesis by examining endothelial cell responses to VEGF.

3.2 Methods

3.2.1 Development of an angiogenesis assay using mouse aortic rings

To obtain an *in vitro* model in which to investigate the influence of glucocorticoids on angiogenesis, extensive methodological development was undertaken. The mouse aortic ring assay was selected to be developed because this model is acknowledged to incorporate most of the components of *in vivo* angiogenesis including cell proliferation, migration and tube formation (Auerbach *et al.* 2003). This model is not technically demanding in terms of tissue preparation, is easy to use to investigate putative angiogenic compounds, lends itself to be used in transgenic models and allows monitoring and quantification of growth. The intention through assay refinement was to produce an aortic ring angiogenesis *in vitro* assay that provided sufficient quantifiable growth in a short space of time that would allow the study of inhibitors of new vessel formation. It was important in establishing the assay to select media and supporting matrix conditions to promote growth without the use of excessive exogenous growth factors that might mask the influence of angiostatic compounds. In addition, because of the subject of this thesis it was important to determine the direction of 11βHSD activity in intact aortic rings in culture.

Using modifications of protocols from rat (Nicosia & Ottinetti 1990) and mouse (Rohan *et al.* 2000) angiogenesis models, several different incubation conditions,

time courses and methods of quantification were tested before a satisfactory experimental protocol was established. These are outlined in the following section.

3.2.1.1 Aortic ring preparation (See section 2.4.)

In these studies 10-12 weeks old C57Bl6 and homozygous null 11β-HSD 1 (bred to be congenic) male mice were used. Animals were killed by schedule 1 methods and thoracic aortae were removed, washed in serum free MCDB 131 medium (Invitrogen, UK), cleaned of periadventitial tissue and divided into 1-3 mm rings.

3.2.1.2 Culture medium

Aortic rings were incubated in MCDB-131 a specially formulated endothelial cell medium developed for endothelial cell growth in a low serum environment (Knedler & Ham 1987). Previously this medium had been used in conjunction with the EGM-2 Bullet kit of growth factors (Clonectics Biowhittaker, UK) to promote endothelial cell growth (Rohan *et al.* 2000). Whilst accurate concentrations of factors with the EGM-2 kit are unavailable from the company because of patent constraints, details regarding the final concentration range of most of the growth factors have been obtained (Table 3.1).

Preliminary experiments established the optimum medium for angiogenesis and defined the standard media conditions to be used in subsequent experiments this comprised: modified MCDB-131 supplemented with EGM-2 aliquots ascorbic acid, heparin and GA1000 as described in section 2.4. Under these conditions sufficient angiogenesis occurred to allow accurate quantification and investigation of the angiostatic effects of glucocorticoids.

Growth Factor	EGM-2 Bullet Kit Concentration range	Retained in standard culture conditions
Heparin	1-5μg/ml	YES
Ascorbic acid	1100mg/ml	YES
GA1000	Not disclosed	YES
IGF-1	5-50ng/ml	Not retained
VEGF	1-5μg/ml	Not retained
hFGF	5-20ng/ml	Not retained
hEGF	5-10ng/ml	Not retained
Hydrocortisone	Not disclosed	Not retained
FBS	Not disclosed	Not retained

Table 3.1 Contents of EGM-2 Bullet Kit

Bio-Whittaker UK supplied the concentrations of bullet kit components under patent constraints. Heparin, ascorbic acid and GA 1000 were retained to supplement MCDB-131 in standard culture conditions. IGF-1 human recombinant insulin like growth factor-1, VEGF vascular endothelial growth factor, hFGF human fibroblast growth factor, hEGF human epidermal growth factor, FBS fetal bovine serum.

3.2.1.3 Embedding Matrix

Matrigel a commercially available collagen matrix was used to support angiogenic growth from the aortic rings. Matrigel is produced in a standard formulation, (Matrigel) and in a growth factor reduced (GFR-Matrigel) form (Table 3.2). Both preparations are steroid free as a consequence of a dialysis step in their purification processes (Communication from Bio-Whittaker). In preliminary experiments a comparison was made of the angiogenic response of aortic rings embedded in Matrigel versus GFR-Matrigel. As a result of these experiments standard Matrigel was used in subsequent investigations.

Aortic rings embedded in Matrigel and incubated in standard media conditions developed numerous outgrowths resembling vessels. The outgrowths first appeared from the second day in culture and continued to develop at an exponential rate over the first week. Media was changed every 48 hours, and experiments were performed in triplicate.

3.2.1.4 Characterisation of Aortic ring outgrowths

The endothelial nature of outgrowths was determined by DiI fluorescently labelled acetylated low-density lipoprotein (DiI-Ac-LDL) (Biomedical technologies, USA) as detailed in Section 2.5.4. Initially 50µl of the stock 200µg/l DiI-Ac-LDL was incubated with a sample aortic ring in 1ml of medium for 4 hours according to manufacturers instructions. A second protocol extending the incubation time to 48 hours was used to optimise cellular up-take of fluorescent probe.

Growth factor	Range of concentration in Matrigel matrix	Average growth factor concentration in Matrigel matrix	Typical concentration in GFR Matrigel matrix
EGF	0.5-1.3ng/ml	0.7ng/ml	<0.5ng/ml
bFGF	<0.1-0.2pg/ml	n.a.	n.d.
NGF	<0.2ng/ml	n.a.	<0.2ng/ml
PDGF	5-48pg/ml	12pg/ml	<5pg/ml
IGF-1	11-24ng/ml	16ng/ml	5ng/ml
TGF-β	1.7-4.7ng/ml	2.3ng/ml	1.7ng/ml

Table 3.2 Amounts of growth factors present in Matrigel versus Growth factor reduced Matrigel (GFR-Matrigel)

The values are supplied by manufacturer on the product information details. EGF epidermal growth factor, bFGF basic fibroblast growth factor, NGF nerve growth factor, PDGF platelet derived growth factor, IGF-1 insulin like growth factor –1, TGF-β Transforming growth factor beta.

n.a.-not applicable, n.d.- not determined.

3.2.1.5 Quantification and time course of angiogenesis

Quantification of growth was performed with direct light microscopy and by image analysis (For imaging details see section 2.4.5). At light microscopy (x50) new vessels were counted using the methodology described by Nicosia et al (Rohan *et al.* 2000). In addition to this established method a variety of assessments were used to determine whether the accuracy and consistency of quantification could be improved. Additional assessments of angiogenesis were performed using computer-assisted analysis of photographed aortic ring cultures. Images were captured from a live feed camera and assessed using The Microcomputer Imaging Device (MCID) (Imaging Research Inc, Canada). MCID measured area of growth, maximum and average diameter of growth and vessel density. The vessel density measurement was derived using computer analysis of pixel density (vessels can be distinguished from background using colour adaptation and the number of colour pixels divided by the area of growth).

New vessel counting by direct light microscopy (x50) was used to assess the time course of the angiogenic response. New vessels were seen from days 2-3 in culture. The rate of new vessel growth achieved a plateau at day 7 and in subsequent experiments angiogenesis was measured at this time point.

3.2.2 Influence of glucocorticoids on angiogenesis in the aortic ring assay

Prior to embarking on investigations to explore the influence of glucocorticoids as putative inhibitors of angiogenesis, the model was used to examine the effects of recognised angiogenesis inhibitors under these conditions.

3.2.2.1 Nitric Oxide and prostanoid influence on angiogenesis *in vitro*

Studies were performed to examine the angiogenic influence of endothelial mediators nitric oxide and prostanoids by incubating aortic rings from C57Bl6 mice in the presence or absence of the nitric oxide synthase inhibitor N^G-nitro-L-arginine; (LNNA) (10^{-4}M) (Alderton *et al.* 2001) or the cyclo-oxygenase inhibitor indomethacin (10^{-5}M) (Leyssac *et al.* 1975) in standard incubation conditions.

3.2.2.2 Influence of glucocorticoids on angiogenesis *in vitro*

Aortic rings from C57Bl6 and homozygous null 11β-HSD 1 mice were obtained as described in section 2.1.2. The angiostatic effect of physiologically relevant concentrations of glucocorticoids was examined by incubating aortic rings under standard conditions in the presence and absence of corticosterone (3nM, 30nM, 300nM, 600nM) and 11dehydrocorticosterone (300nM, 600nM). As steroids had been dissolved initially in ethanol and then diluted into aqueous solutions, vehicle control conditions were established that contained equivalent aqueous dilutions of ethanol. Vehicle or steroid was added to the culture medium at the start of the experiments and media replaced every 48 hours. Experiments were performed in triplicate; paired concomitant controls were performed for each set of conditions.

3.2.2.3 Identification of the receptor responsible for glucocorticoid-induced angiostasis

Aortic rings from C57Bl6 mice were incubated in standard conditions in the presence of the endogenous mineralocorticoid aldosterone (10^{-8}M) or the mineralocorticoid receptor antagonist spironolactone ($10^{-5}$and 10^{-6}M) or the glucocorticoid receptor antagonist, RU38486 (10^{-6}M) or vehicle.

3.2.2.4 Influence of 11βHSD-1 on the angiostatic effect of glucocorticoids

A dual approach of pharmacological inhibition and transgenic deletion was adopted to investigate whether 11βHSD-1 in the aortic wall influenced the effects of glucocorticoids to modulate angiogenesis. Both pharmacological inhibition of 11βHSDs with carbenoxolone and 11βHSD-1 transgenic deletion were used.

Pharmacological inhibition of 11βHSDs- Aortic rings from C57Bl6 mice were incubated in standard conditions with corticosterone (600nM) or 11dehydrocorticosterone (600nM) in the presence of the non-selective 11βHSD inhibitor carbenoxolone (CBX; 10^{-4}and10^{-6}M) or vehicle.

Transgenic deletion of 11βHSD1 - Aortic rings from C57Bl6 and homozygous null 11βHSD1 (-/-) mice were incubated with corticosterone (600nM) or 11dehydrocorticosterone (600nM) using standard conditions.

3.2.3 11βHSD activity assay in intact aortic rings

To determine the direction of 11βHSD activity in intact aortic rings in vitro a method (Souness *et al.* 2002) was adapted by Dr Anna Dover and Mrs Eileen Miller.

Aortic rings were collected as described previously in section 2.4. and cultured for 24 hours in DMEM-F12 medium (Invitrogen, UK) supplemented with fetal bovine serum (1%), streptomycin (100μg/ml), penicillin (100units/ml) and amphotericin (0.25μg/ml) and 11βHSD activity was established by the conversion of ^{3}H-steroid. 11β-Reductase activity was determined by adding 10pmol [$^{3}H_4$]-11-dehydrocorticosterone. Mouse liver and medium alone were used as controls. 11β-Dehydrogenase activity was determined by adding 10pmol 1,2,6,7-[$^{3}H_4$]-corticosterone. Mouse kidney slices and medium alone served as positive and negative controls.

After incubation, steroids were extracted from media; [$^{3}H_4$]-Corticosterone and [$^{3}H_4$]-11-dehydrocorticosterone were separated by HPLC and quantified by on-line liquid scintillation counting (Walker *et al.* 1991). Enzyme activity was expressed as conversion after subtraction of apparent conversion in negative control wells.

3.2.4 The influence of glucocorticoids on calcium signaling in endothelial cells

A possible mechanism for glucocorticoid-induced inhibition of angiogenesis was investigated by assessing the intracellular calcium response to VEGF in the presence of glucocorticoids or vehicle. Please refer to section 2.6.

3.2.4.1 Human Umbilical Vein Endothelial cell culture

Confluent monolayers of endothelial cells at passage 4 on fibronectin coated glass coverslips (a gift from Miss Catherine Shaw) were incubated in serum free conditions. Cells were loaded with Fura-2AM (5μM) and incubated with vehicle or cortisol (16μM, 600nM) for 6 hours and placed on the stage of an inverted fluorescence microscope. For each experiment baseline fluorescence was established followed by the addition of 10ng/ml (Faehling *et al.* 2002) human recombinant $VEGF_{A165}$ (Sigma, UK). Data were acquired for 15 minutes for preliminary experiments (Faehling *et al.* 2002); in later work this was reduced to 8 minutes to reflect the time scale of the response.

The ratio of intensities obtained from 340/380 excitation pairs was used as a measure of $[Ca^{2+}]i$ (Grynkiewicz *et al.* 1985). Calibration of the $[Ca^{2+}]i$ was performed by monitoring the change in fluorescence at minimal and maximal $[Ca^{2+}]i$ responses as detailed in section 2.6.2.

3.2.5 Statistics

Data are mean ± SEM. "n" refers to the number of different mice, where possible all measurements of angiogenesis *in vitro* were made in triplicate. Comparisons were made by ANOVA with least squares difference post hoc tests. Inter-assay- and intra-assay coefficients of variation were 17 % (n=32) and 22 % (n=18), respectively for vessel number in aortic rings after 7 days in culture.

3.3 Results

3.3.1 Development of an angiogenesis assay using mouse aortic rings

Culture Medium- Initial experiments indicated that incubation of embedded aortic rings in culture medium alone without EGM-2 bullet kit did not promote sufficient spontaneous angiogenesis to measure the effect of an angiostatic compound (data not shown).

Further experiments were performed using the bullet kit and comparing angiogenesis in the presence or absence of FBS. This work indicated that FBS inclusion produced excessive growth that could not be quantified beyond 3 days of incubation. Owing to the inability to quantify angiogenesis under these conditions this particular series of incubations was discontinued after two experiments. In the absence of FBS a vigorous angiogenic response was observed that could be quantified however in these conditions physiologically relevant concentrations of glucocorticoids did not influence angiogenesis (Figure 3.1). Further method refinement investigated the effects of glucocorticoid on angiogenesis in the presence of heparin, ascorbic acid and GA1000 without VEGF, IGF-1, hEGF and hFGF. Under these conditions growth of new vessels was quantifiable and sufficient, 100-150 new vessels were counted after 7 days in culture (Figure 3.4). In addition in the presence of glucocorticoids (300nM) angiogenesis was impaired. Therefore these conditions were adopted as standard incubating media in all further studies (see Table 3.1).

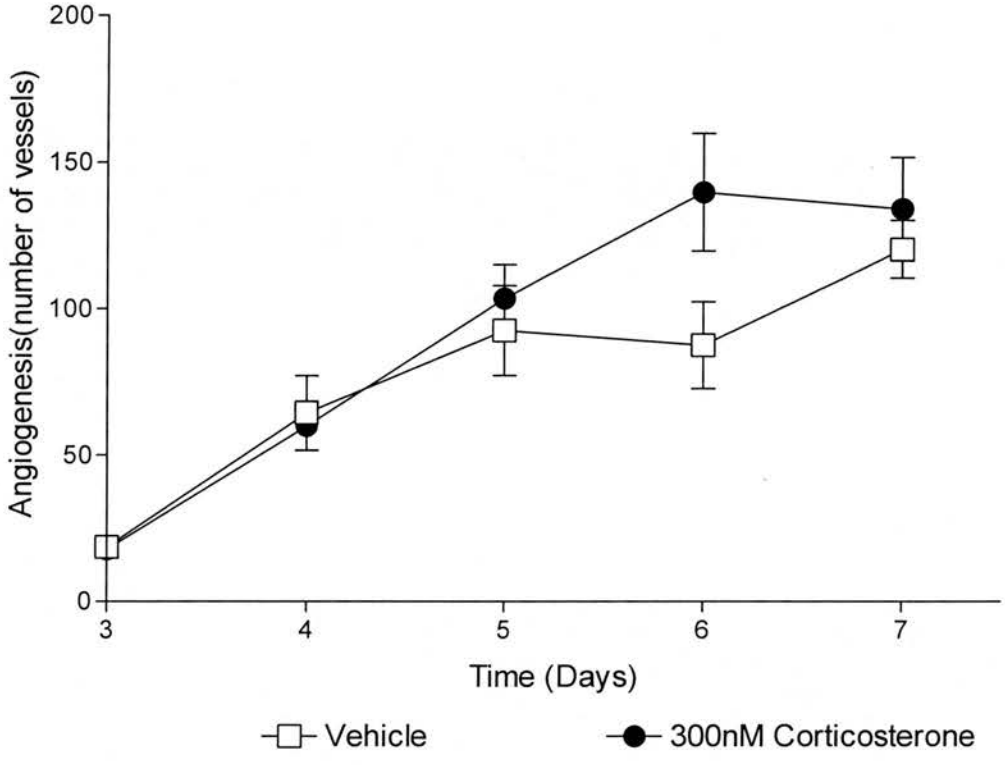


Figure 3.1 Angiogenesis in mouse aortic rings: the influence of glucocorticoids in the presence of exogenous growth factors

C57Bl6 aortic rings incubated in MCDB131 (supplemented with EGM-2 bullet kit excluding hydrocortisone and FBS) in the presence (solid circle) or absence (open squares) of corticosterone. Angiogenesis was quantified as the number of vessels. Data represent mean ± SEM (n = 6) no significant differences between conditions were detected using two-way ANOVA.

3.3.1.1 Embedding Matrix

Initial observations using standard Matrigel had raised concerns regarding the prolific nature of the angiogenic response compared with previous studies (Rohan *et al.* 2000; Nicosia & Ottinetti 1990). Similar vigorous angiogenic responses were seen with either standard or GFR Matrigel matrix, (data not shown). In light of initial results with standard Matrigel, and practical difficulties with obtaining GFR Matrigel, standard Matrigel was used throughout.

3.3.1.2 Characterisation of Aortic ring outgrowths

Using the manufacturers recommended incubation time of 4 hours DiI-Ac-LDL did not label aortic ring outgrowths. Extension of the incubation time to 48hours resulted in fluorescent probe uptake by both cells within the aortic ring and by vascular outgrowths (Figure 3.2)

3.3.1.3 Assay variability

Inter-assay coefficients of variation for vessel counts on day 7 in control aortic rings was measured by calculating the mean, standard deviation and SEM for all controls (n=32) and using these figure performing the function (SEM/standard deviation)*100. The inter-assay variation was calculated to be 17 % (n=32 mice). Intra-assay variation for vessel counts on day 7 in control aortic rings was assessed by calculating the co-efficient of variation of experiments using the same animal but performed on different culture plates (n=18 mice). The mean of these values was taken as the intra-assay variation and calculated as 22 % (n=18).

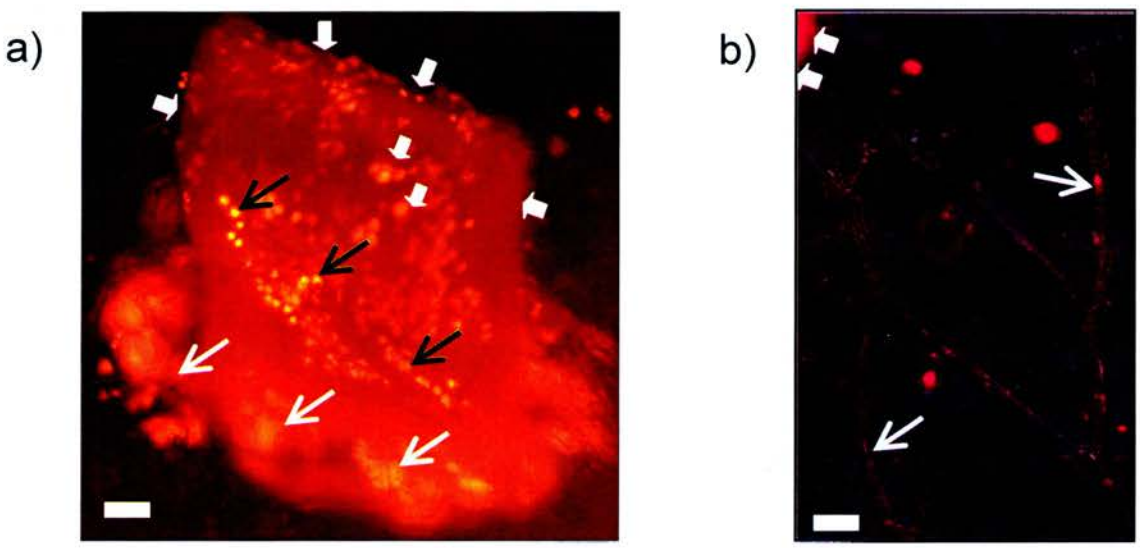


Figure 3.2 Characterisation of aortic ring outgrowth by uptake of DiI-Ac-LDL shown by fluorescence microscopy

C57Bl6 aortic ring incubated for 7 days in standard conditions in the absence of glucocorticoids (a) low power (x50) magnification (white scale bar is 0.2 mm) demonstrating aortic ring explant (thick white arrows) with labelled endothelial cells (black arrows) and out growths similarly with labelled endothelial cells (thin white arrows) (b) Aortic ring outgrowth at high magnification (x100) thin white arrows indicate endothelial cells, aortic ring explant can also be seen (thick white arrow) (white scale bar is 0.02mm).

3.3.1.4 Quantification and time-course of angiogenesis

Under standard incubation conditions (see section 2.1) wild type aortic rings sprouted new vessels from days 2-3. Subsequently new vessel growth occurred exponentially from day 3 to day 7 then reached a plateau (Figure 3.3). Although the longevity of new vessel survival was not formally tested, on one occasion vessels were kept for 23 days without deterioration.

Methods for the quantification of angiogenesis were assessed with regard to their reliability, reproducibility and consistency. Computer generated measurement of vessel density proved to be an unreliable and inconsistent method of measurement and was discontinued. Quantification of angiogenesis by total growth area, average diameter or maximum diameter was comparable to quantification by vessel counting as assessed by measuring the angiogenesis response in the presence of glucocorticoid. All 4 methods detected similar patterns of new vessel growth; all detected inhibition of angiogenesis by glucocorticoids and the degree of inhibition was comparable across all parameters (Figure 3.4 a-d). Vessel counting was technically less demanding, required only the use of light microscopy, was reliable and the most widely used parameter of angiogenesis in the literature (Rohan *et al.* 2000; Nicosia & Ottinetti 1990) therefore subsequent quantification of angiogenesis was performed by this method.

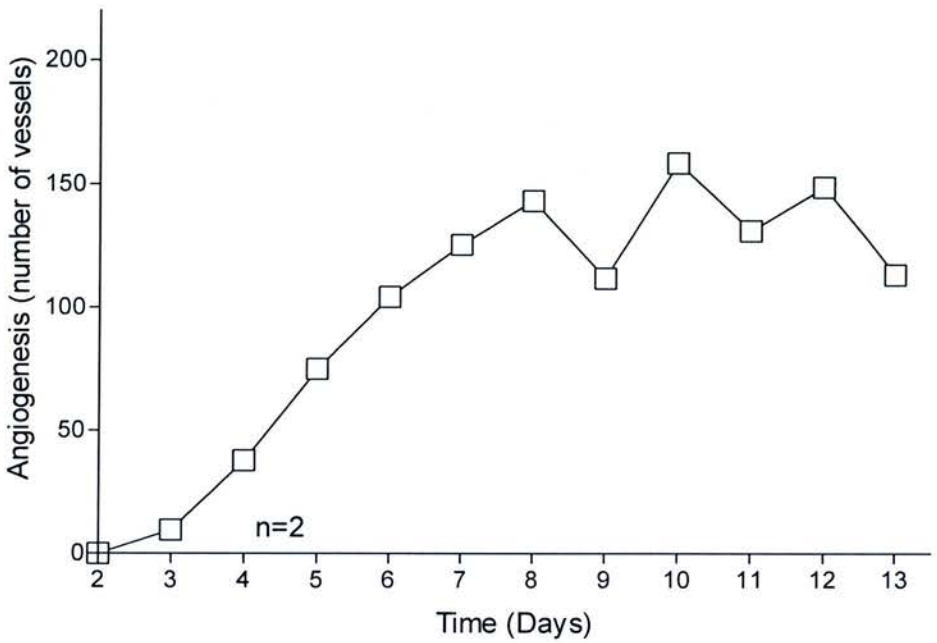


Figure 3.3 Time course of angiogenesis.

Results from C57Bl6 aortic rings incubated with vehicle in standard conditions. Preliminary experiments found new vessels present from day 2-3 in culture; growth occurred exponentially from day 3 to day 7 then reached a plateau. Data are mean for n=2

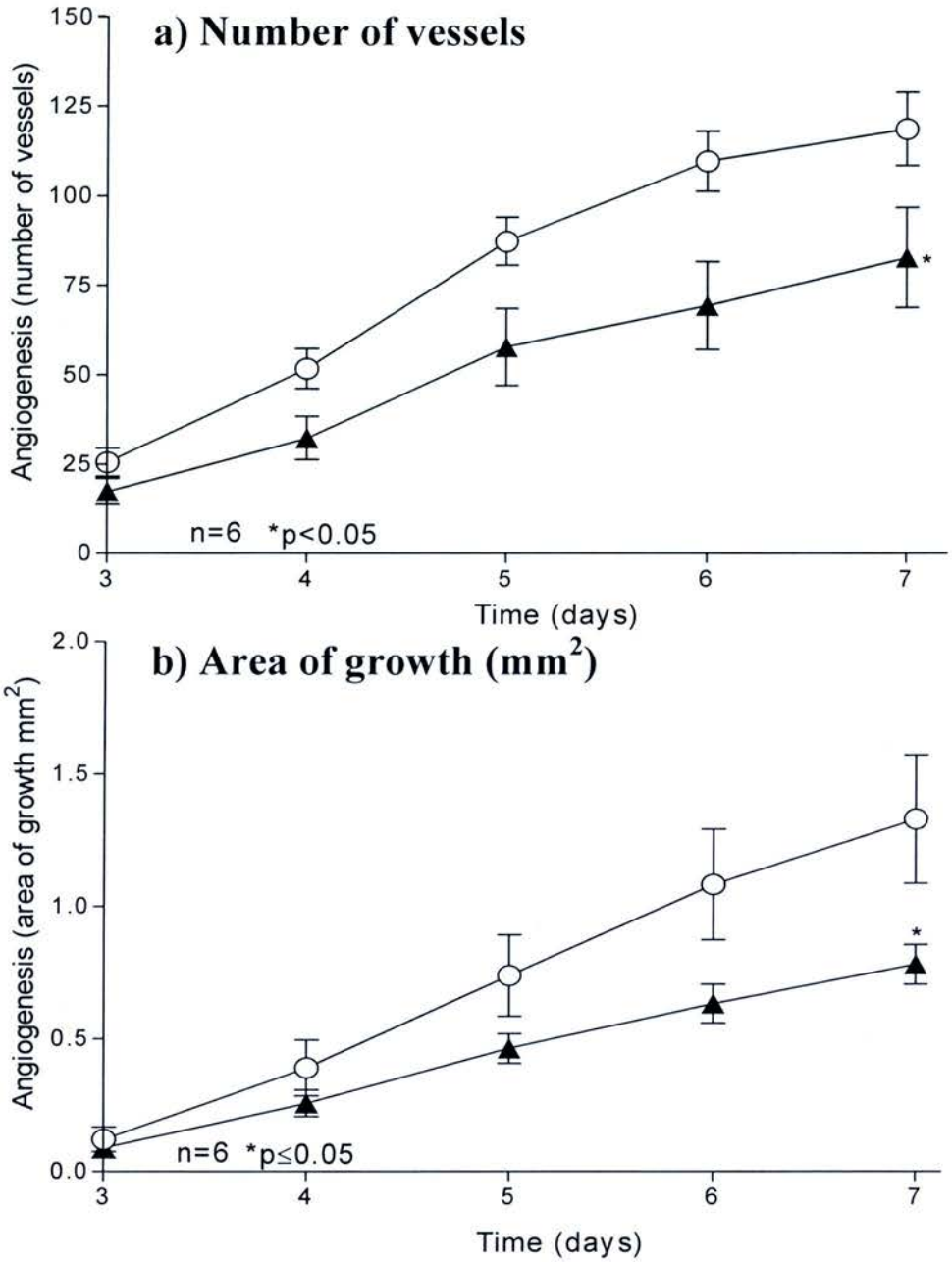


Figure 3.4 (a-b) Comparison of quantification methods using the angiostatic effects of glucocorticoids

Wild type aortic rings incubated in standard conditions in the presence of vehicle (open circles), corticosterone (300nM) (solid triangles). Glucocorticoids inhibited angiogenesis consistently using 4 methods of quantification: (a) number of vessels, (b) total area of new growth. Data are expressed as means ± SEM. *p≤ 0.05, n=4-6.

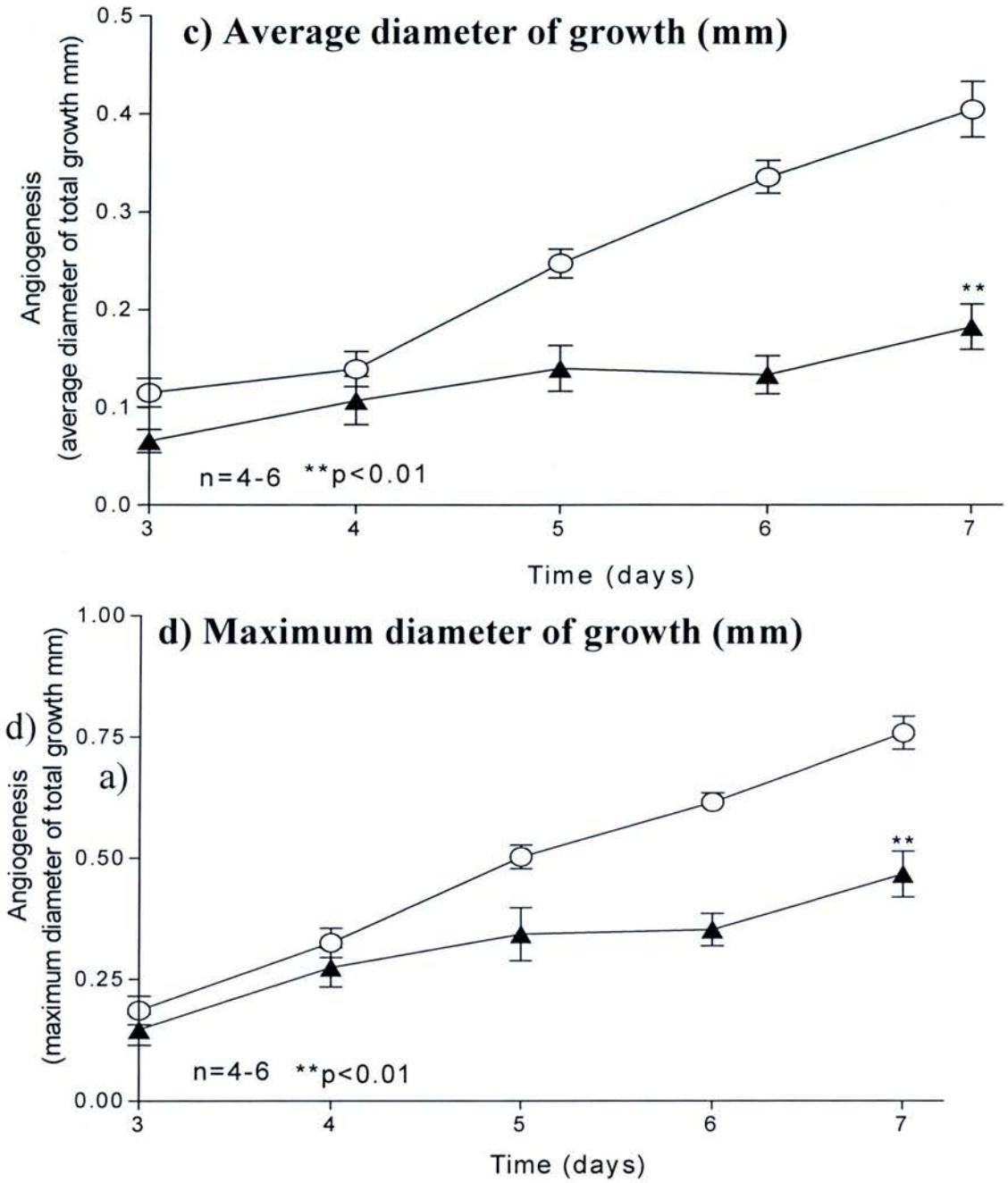


Figure 3.4(c-d) Comparison of quantification methods using the angiostatic effects of glucocorticoids (continued)

Wild type aortic rings incubated in standard conditions in the presence of vehicle (open circles), corticosterone (300nM) (solid triangles). Glucocorticoids inhibited angiogenesis consistently using 4 methods of quantification: (c) average and (d) maximum diameters of total growth. Data are expressed as means ± SEM. **p≤ 0.01, n=4-6.

3.3.2 Influence of glucocorticoids on angiogenesis in the aortic ring assay

3.3.2.1 Nitric Oxide and prostanoid influence on angiogenesis in vitro

Following 7 days in culture angiogenesis was impaired in aortic rings incubated with L-NNA (107.4 ± 4.5 vessels) but not in those incubated with indomethacin (157.1 ± 14.3 vessels) compared to vehicle (153.2 ± 17.0 vessels; n=6; $p<0.03$) (Figure 3.5).

3.3.2.2 Influence of glucocorticoids on angiogenesis in vitro

Incubation of aortic rings in the presence of glucocorticoids at concentrations across the physiological range (30-600nM) inhibited angiogenesis (Figure 3.6). A threshold for this response was found to be ≈ 3nM corticosterone. At similar concentrations (300-600nM) the inert 11-keto compound 11dehydrocorticosterone, a substrate for 11βHSD-1 also impaired angiogenesis (Figure 3.6).

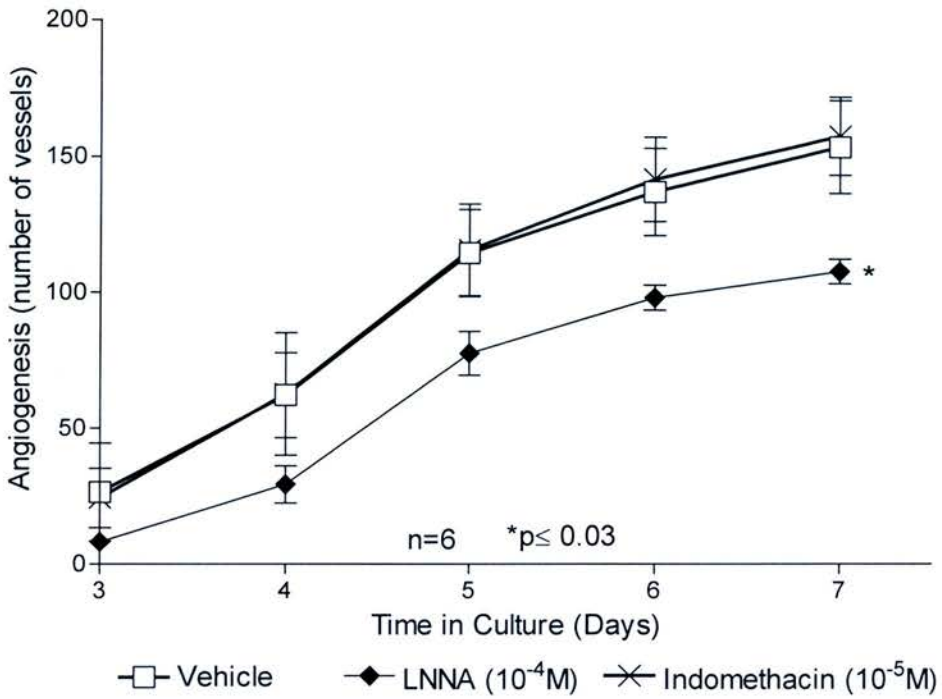


Figure 3.5 Nitric Oxide and prostanoid influence on angiogenesis *in vitro*

Angiogenic responses in aortic rings from wild type mice incubated in the presence of vehicle (open square) or LNNA (10^{-4}M) (solid diamonds) or indomethacin (10^{-5}M) (line crosses). In comparison to vehicle treated aortic rings angiogenesis was inhibited by the presence of LNNA but was not influenced by indomethacin (* p≤0.03 vs vehicle Comparison was by repeated measures ANOVA). Data points are mean ± SEM (n=6).

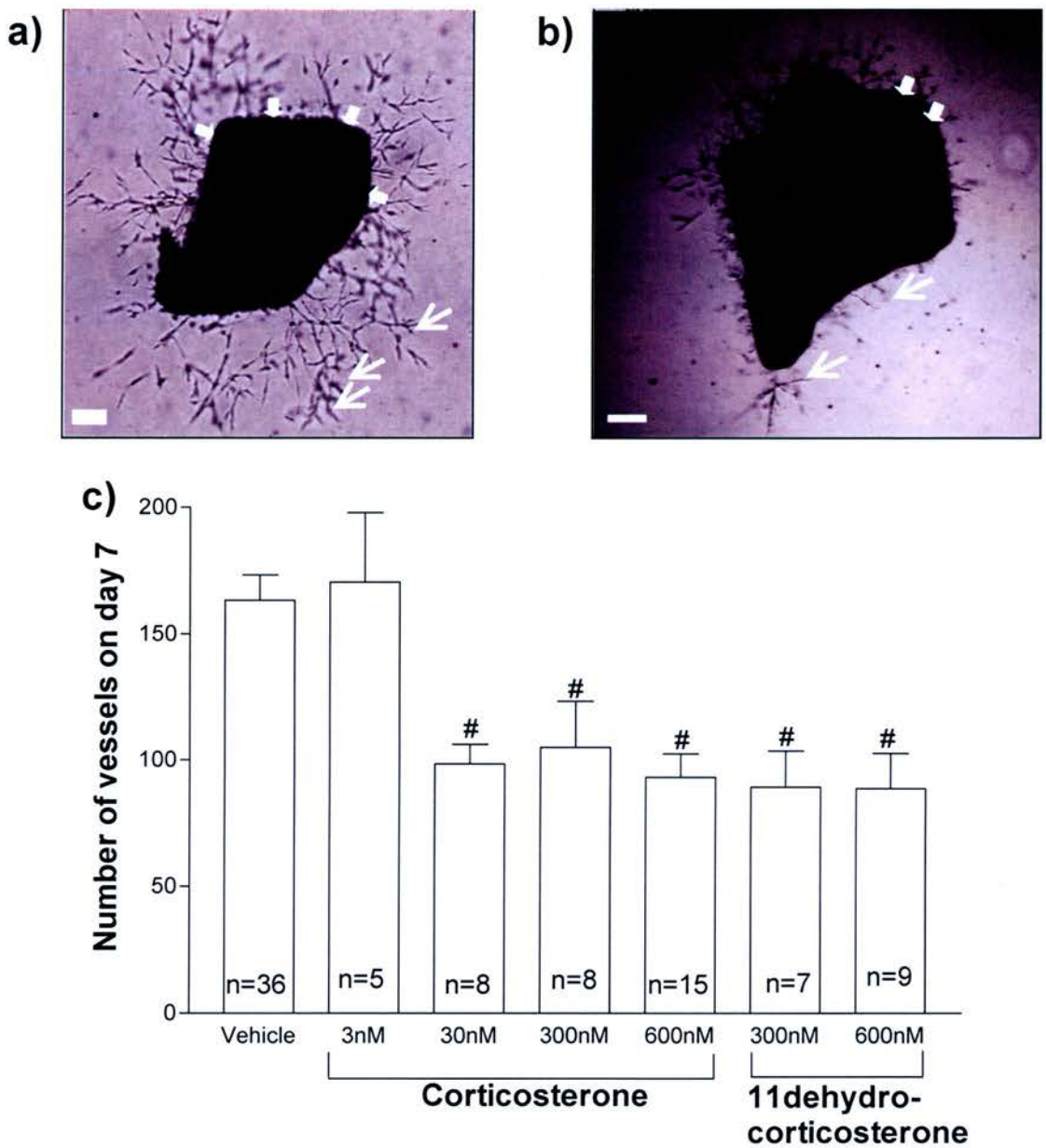


Figure 3.6 Influence of physiologically-relevant concentrations of glucocorticoids on angiogenesis

(a) Aortic ring incubated for 7 days without glucocorticoid. (b) Aortic ring incubated for 7 days in the presence of corticosterone (600nM) (white scale bar is 0.2 mm). Thick white arrows indicate the aortic ring; thin white arrows indicate new vessels. (c) Aortic rings from C57Bl6 mice incubated in the presence of glucocorticoids. Corticosterone or 11dehydrocorticosterone inhibited angiogenesis. A threshold for the influence of corticosterone was 3nM. Data are expressed as mean ± SEM #p≤ 0.01.

3.3.2.3 Receptor dependency for glucocorticoid induced angiostasis

The glucocorticoid receptor antagonist RU38486 did not influence angiogenesis in comparison to vehicle but eliminated the angiostatic effects of both corticosterone and 11-dehydrocorticosterone (Figure 3.7).

High concentrations of mineralocorticoid antagonist spironolactone (10^{-5}M) (66.4 ± 24.7 vessels) impaired angiogenesis in comparison to vehicle (185.6 ± 17.8 vessels) (n=6, $p<0.001$). At a lower concentration (10^{-6}M) angiogenesis was not affected by spironolactone and this concentration did not alter the angiostatic effects of corticosterone or 11-dehydrocorticosterone (Figure 3.7). In addition aldosterone (10^{-8}M) did not influence angiogenesis ((157.3 ± 28.0 vessels) versus vehicle (185.6 ± 17.8 vessels) n=6).

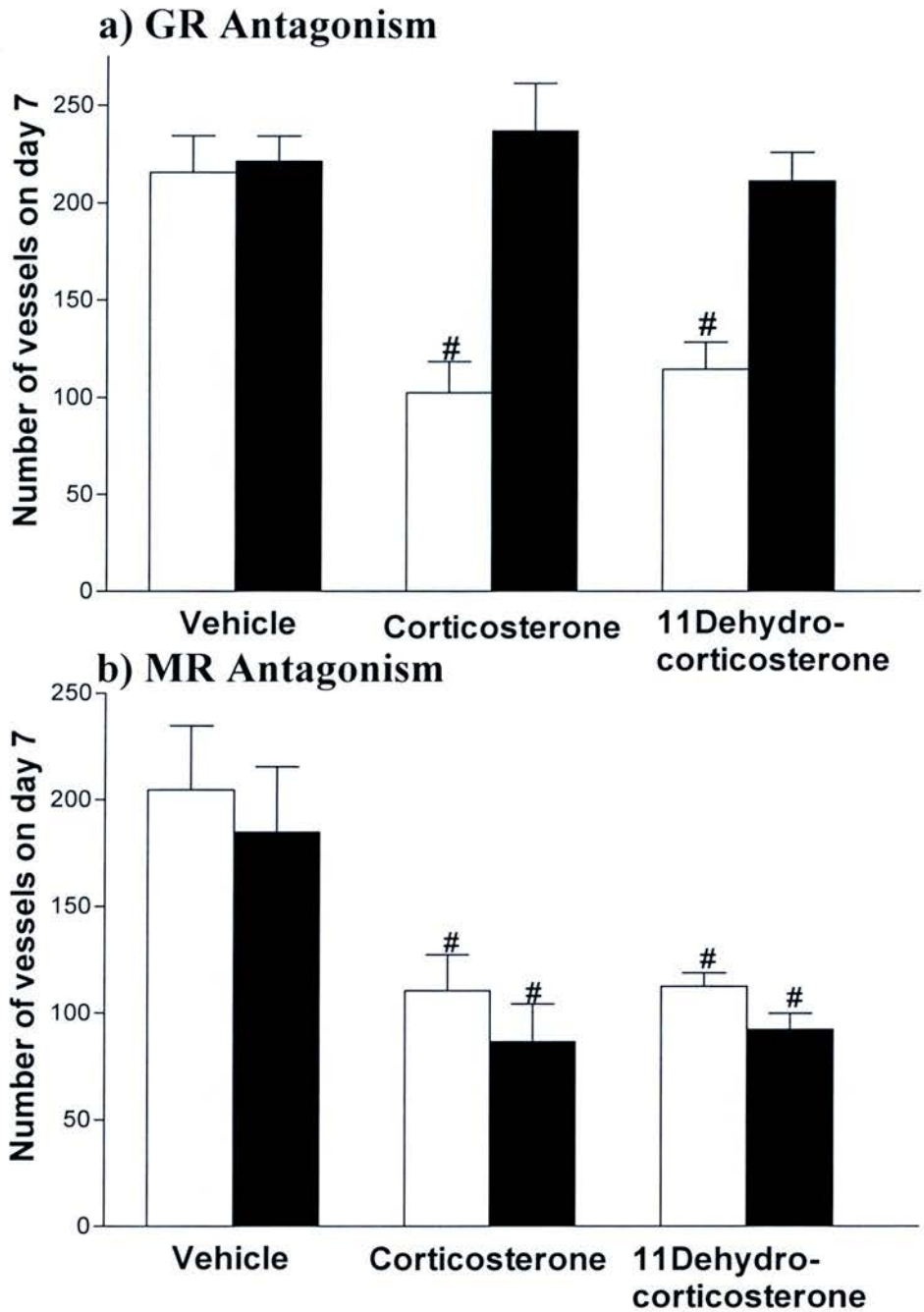


Figure 3.7 Glucocorticoid-induced angiostasis is GR dependent

Angiogenic responses of aortic rings from C57Bl6 mice (a) Incubated with (filled columns) or without (open columns) RU38486 (10^{-6} M) and glucocorticoids (600 nM). Results are mean ± SEM for n=4-6 experiments. # $p<0.01$ versus corresponding vehicle. RU38486 alone had no effect. (b) Incubated with (filled columns) and without (open columns) spironolactone (1μM) and glucocorticoids (600nM). Spironolactone had neither an effect on angiogenesis in comparison to vehicle nor on the influence of glucocorticoids. Results are mean ± SEM for n=6 experiments. #$p<0.02$ versus corresponding vehicle.

3.3.2.4 Influence of 11βHSD-1 on the angiostatic effect of glucocorticoids

Pharmacological inhibition of 11βHSDs- The non-selective 11βHSD inhibitor carbenoxolone at high concentration (10^{-4}M) abolished all growth in aortic ring cultures (data not shown). At a lower concentration (10^{-6}M) carbenoxolone did not inhibit angiogenesis and did not influence the angiostatic effects of corticosterone (600nM). In contrast this concentration of carbenoxolone attenuated the angiostatic effect of 11dehydrocorticosterone (600nM) (Figure 3.8a).

Transgenic deletion of 11βHSD1- Angiogenesis was similar in vehicle-treated control aortic rings from C57Bl6 compared to 11βHSD1 -/- mice. As before corticosterone and 11dehydrocorticosterone inhibited angiogenesis in wild type aortic rings. In contrast 11βHSD1 transgenic deletion abolished the angiostatic effect of 11-dehydrocorticosterone but had no effect on the angiostatic influence of corticosterone (Figure 3.8b).

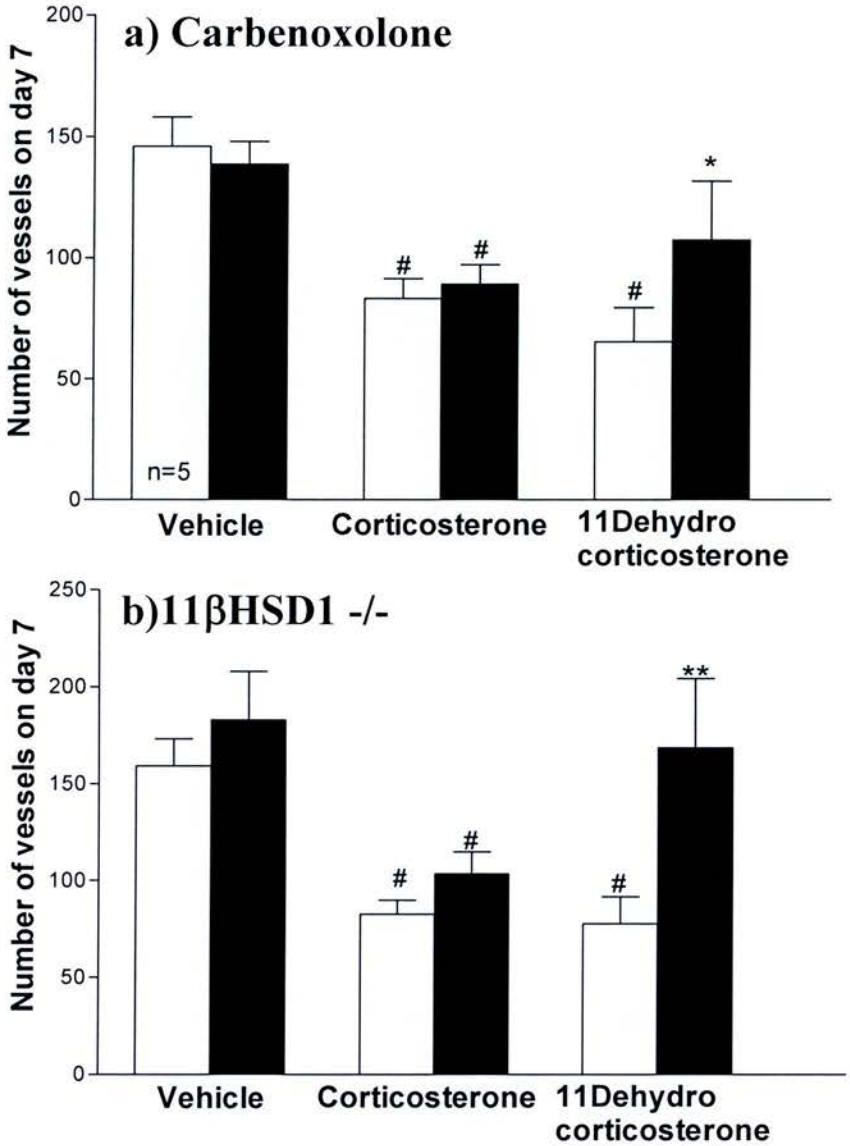


Figure 3.8 (a-b) Influence of 11βHSD-1 on angiogenesis

a) Effect of glucocorticoids (600nM) on angiogenesis in aortic rings from C57Bl6 mice with (filled columns) and without (open columns) carbenoxolone (10^{-6} M). Data are mean ± SEM. n=5 #p<0.01 versus corresponding vehicle. *p<0.04 for the effect of carbenoxolone in the presence of 11-dehydrocorticosterone. Carbenoxolone had no effect in the presence of corticosterone or vehicle alone.

b) Aortic rings from wild type (open columns) or 11βHSD1 -/- (filled columns) mice with and without glucocorticoids (600nM). Data are mean ± SEM n=7 #p<0.01 versus corresponding vehicle. **p<0.01 for differences in angiogenesis between wild type and 11βHSD1 -/- mice. Angiogenesis was not difference between strains in the presence of vehicle or corticosterone but was inhibited by 11dehydrocorticosterone in C57Bl6 but not 11βHSD1 -/- mice.

3.3.3 11βHSD activity in aortic rings *in vitro*

Reductase activity was demonstrated by the conversion of 1,2,6,7-[$^3H_4$]-11-Dehydrocorticosterone to [$^3H_4$]-Corticosterone; dehydrogenase activity was demonstrated by the conversion of 1,2,6,7-[$^3H_4$]-Corticosterone to [$^3H_4$]-11-Dehydrocorticosterone in intact aortic rings and control tissues. Both 11βHSD reductase and dehydrogenase activities were present in intact aortic rings. Results are expressed per mg of wet tissue (Figure 3.9).

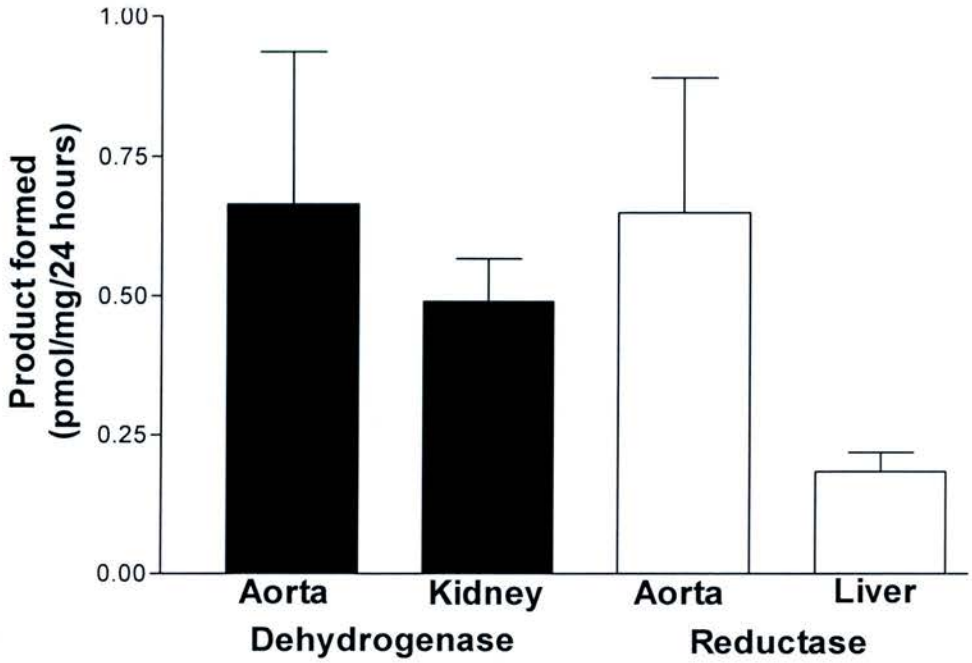


Figure 3.9 11βHSD enzyme activities in aortic rings from wild type mice

Rings were incubated with [$^3H_4$]-corticosterone (dehydrogenase) (solid bars) or [$^3H_4$]-11-dehydrocorticosterone (reductase) (open bars) and conversion to the relevant product measured after 24 hours. Dehydrogenase and reductase activities were detected in aortic rings (*$p<0.04$ vs negative control (=zero by definition), #$p<0.01$). Aortic ring dehydrogenase and reductase activities were not significantly different from equivalent wet weights of kidney or liver, respectively. Results are mean ± SEM (n=4-6).

3.3.4 Glucocorticoid inhibition of endothelial cell signaling *in vitro*

This work was performed as a pilot study to gain an insight into the mechanisms for glucocorticoid induced angiostasis. Consequently the following data represent preliminary findings (n=2-3). To demonstrate the degree of variability in the data intracellular calcium concentrations are presented as the mean ± SEM from individual cells. Results were obtained for 10-15 cells per preparation n=2-3. Therefore data are mean ± SEM for 20-30 values.

3.3.4.1 VEGF induced calcium transients in endothelial cells

$VEGF_{A165}$ (10ng/ml) stimulated a transient biphasic intracellular calcium response in HUVECs. The biphasic response was characterised by an initial sharp peak followed by a second sustained rise (Figure 3.10a) as previously described (Faehling *et al.* 2002b). The time to initial peak was 48.8 ± 1.9 seconds and the duration of the response was 4.67 ± 0.16 minutes.

3.3.4.2 Influence of glucocorticoids on HUVEC intracellular calcium responses to VEGF

Pharmacological concentrations of glucocorticoids- In the presence of pharmacological concentrations of cortisol (16μM) a biphasic response was not observed. The secondary sustained rise seen in the presence of vehicle was abolished in 86% of cells incubated with cortisol (Figure 3.10b). Quantification of intracellular calcium responses from 20-30 cells on 3 coverslips (analysed by repeated measures

ANOVA) indicated that cortisol (16μM) reduced the intracellular calcium response to VEGF ($p<0.001$) (figure 3.11). Total change in intracellular calcium (area under the curve) was reduced by cortisol (Δ Ca^{2+} 224 ± 45 nM minutes) in comparison to vehicle (Δ Ca^{2+} 586 ± 64 nM minutes; $p<0.01$).

Physiologically relevant concentrations of glucocorticoids-In comparison to vehicle cortisol (600nM) did not alter the character of the VEGF $_{A165}$ (10ng/ml) stimulated biphasic intracellular calcium response in HUVECS but did reduce the amplitude (Figure 3.13). Quantification of intracellular calcium responses from 20-30 cells on 2-3 coverslips (analysed by repeated measures ANOVA) indicated that cortisol reduced the intracellular calcium response ($p<0.01$) (Figure 3.12). Total change in intracellular calcium (area under the curve) was also reduced by physiologically relevant concentrations of glucocorticoids (Δ Ca^{2+} was 586 ± 64 nM minutes for vehicle and Δ Ca^{2+} 396 ± 44 nM minutes for cortisol (600nM) n=2-3 coverslips, $p\leq0.03$).

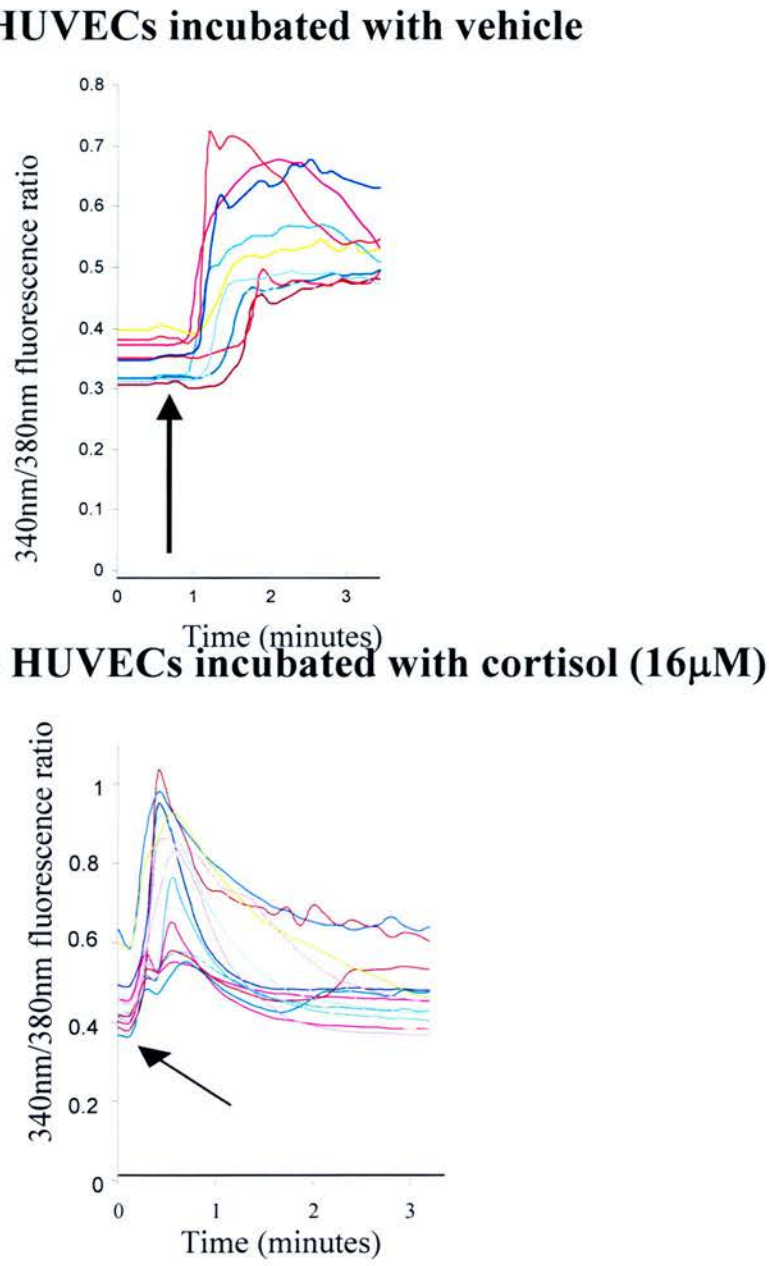


Figure 3.10 Effects of VEGF on HUVEC intracellular calcium transients

Representative traces of the intracellular calcium transients in HUVECs stimulated by $VEGF_{A165}$ (10ng/ml) in a) the presence of vehicle b) In the presence of cortisol 16 μM. The black arrow indicates moment of VEGF administration. Each graph represents traces from 10-15 cells on a single coverslip. A biphasic response was observed in vehicle treated cells, in the presence of cortisol the second plateau phase was reduced.

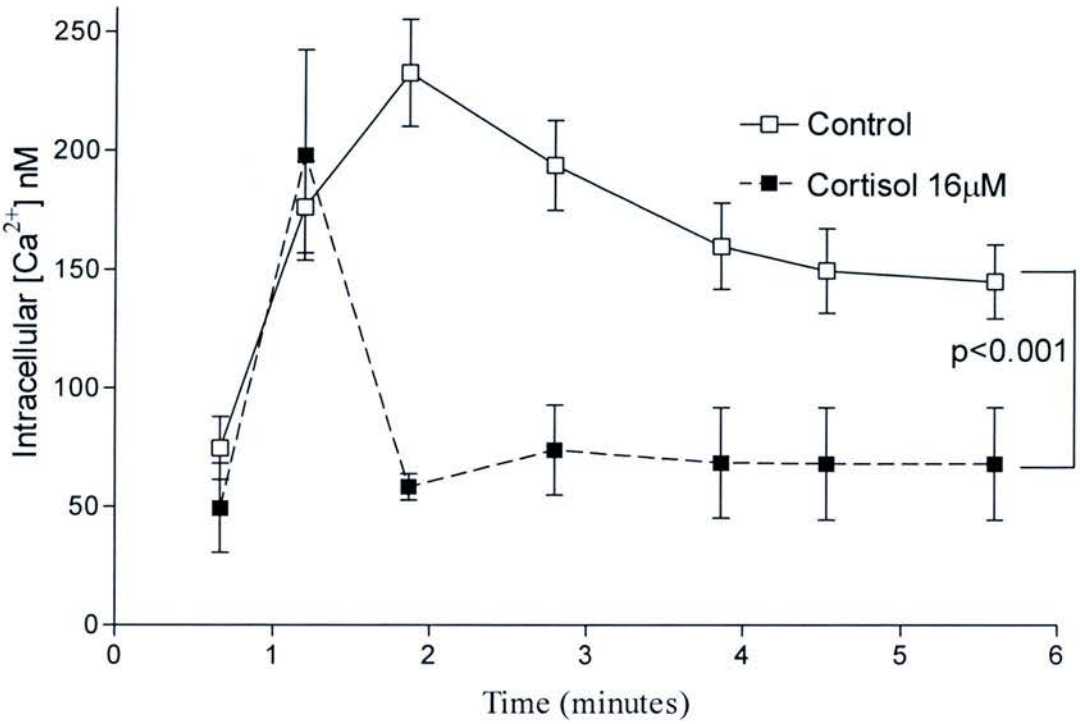


Figure 3.11 Influence of glucocorticoids on HUVEC intracellular calcium responses to VEGF

Intracellular calcium responses in HUVECs to VEGF stimulation in the presence (solid line) or absence (dashed line) of cortisol (16μM). High concentrations of cortisol (16μM) impaired the intracellular calcium response to VEGF stimulation in comparison to vehicle (Data are mean ± SEM intracellular calcium response traces from 20-30 cells on 3 coverslips n=3, *p<0.001 by repeated measures ANOVA).

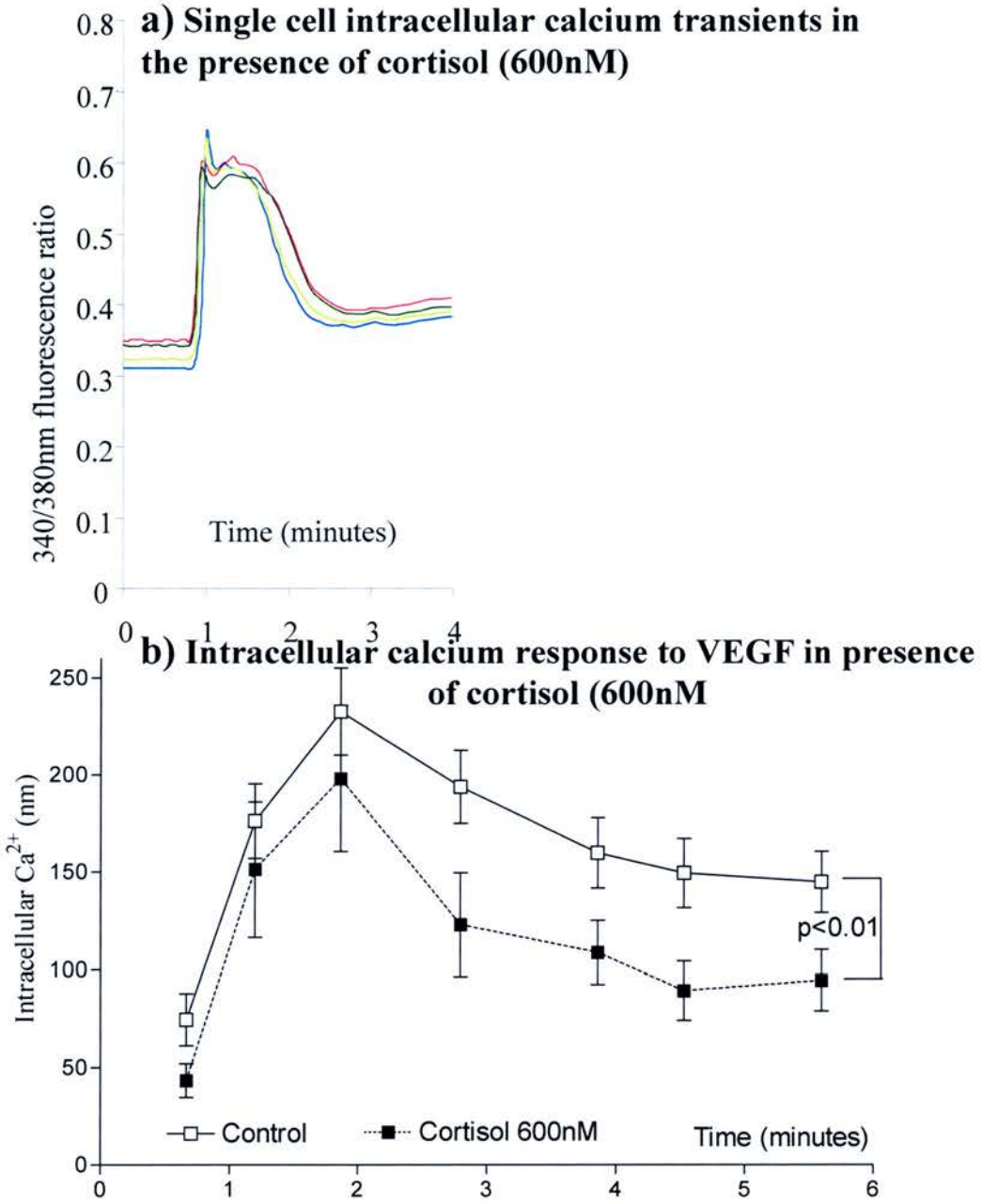


Figure 3.12 Endothelial cell intracellular calcium responses in the presence of physiological concentrations of cortisol (600nm)

(a) Representative traces of the intracellular calcium transients in HUVECs stimulated by $VEGF_{A165}$ (10ng/ml) in the presence of cortisol (600nM). The graph represents traces from 4 cells on a single cover slip. A reduced amplitude biphasic response was observed. **(b)** Intracellular calcium responses to VEGF in the presence (solid line) or absence (dashed line) of cortisol (600nM). Cortisol reduced VEGF induced intracellular calcium transients (Data are mean ± s.e.m from 20-30 cells on 2-3 coverslips, *p<0.01 by repeated measures ANOVA).

3.4 Discussion

In this chapter through the development of an *in vitro* angiogenesis assay using mouse aortic rings it was determined that physiologically relevant concentrations of glucocorticoids inhibit angiogenesis in a glucocorticoid receptor dependent manner. Furthermore local metabolism of glucocorticoids by 11βHSD1 was demonstrated in the vessel wall using intact aortic rings and 11dehydrocorticosterone, a substrate for 11βHSD1 which does not activate corticosteroid receptors, also impaired angiogenesis in a glucocorticoid receptor dependent manner but only in the presence of 11βHSD1. Therefore 11βHSD1, through the regeneration of active glucocorticoids, was found to amplify the angiostatic effects of steroids *in vitro.*

3.4.1 Adaptation of the mouse aortic ring assay

From the available *in vitro* angiogenesis assays the mouse aortic ring model was selected for it combines many of the components of angiogenesis that are represented individually in other cell culture models. Thus the mouse aortic ring model includes elements of cell proliferation and migration; tube formation, branching, remodelling and luminisation. Reviewers have acknowledged the value of incorporating all these facets of angiogenesis into a single assay and recognised that the aortic ring *in vitro* assay most closely resembles *in vivo* physiology (Auerbach *et al.* 2003; Staton *et al.* 2004). Importantly for this thesis the model could also be used with rodent tissues and thus enable the use of genetically modified mouse aortae for example from 11βHSD1-/- mice. Glucocorticoids had not been used in conjunction with the model in mice but had been applied to rat aortic rings in a collagen-based matrix (Nicosia & Ottinetti 1990) and under these conditions hydrocortisone 1μg/ml was angiostatic.

A recognised disadvantage of the model is that nascent new vessels are generated from the aorta, a large conduit artery (Staton *et al.* 2004) whereas *in vivo* angiogenesis occurs through vascular remodelling at a microvascular level (Conway *et al.* 2001). How the large artery vessel wall gives rise to capillary outgrowths has also been questioned (Nicosia *et al.* 1992) and whether the outgrowths derive from luminal endothelial cells, adapted mural-periendothelial cells or from vasovasorum is unclear (Akita *et al.* 1997). It would appear that a degree of cellular transdifferentiation or un-differentiation is required for promoting new vessel growth from aortic ring explants (Akita *et al.* 1997; Nicosia *et al.* 1992). Human embryonic aortae contain pluripotent VEGF receptor 2 (VEFGR2/Flk1) positive cells that are capable of differentiating into endothelial or mural cells in response to VEGF or platelet derived growth factor (PDGF-BB) raising the possibility that a component of the de novo formation of new vessels in aortic rings is from a vascular progenitor stem cell (Iurlaro *et al.* 2003; Akita *et al.* 1997). Although these pluripotent cells have not been isolated in rodent studies, the endothelium in rat aortae has been shown to exhibit considerable plasticity to differentiate into a microvascular angiogenic endothelium and mural cells (Nicosia *et al.* 1992). The mural cells of aortic ring outgrowths display mixed cell markers of vascular smooth cells and endothelial cells and these so called mural precursor cells support the developing endothelial tubes through angiopoietin signalling via Tie-2 rather than via PDGF chemotactic recruitment (Iurlaro *et al.* 2003; Villaschi *et al.* 1994). Despite the controversies surrounding the origin of the endothelial and mural cells of the aortic ring outgrowths, these recent studies confirm the initial electron microscopy studies that identified new vessel growth from aortic rings to consist of endothelial lined

luminised tubes that were covered in mural supporting cells (Nicosia & Ottinetti 1990).

In the study presented in this chapter cells labelled with DiI-Ac-LDL were present on the aortic explant distributed in a pattern consistent with luminal endothelial cells. In addition cells were also labelled with the fluorescent probe on outgrowths scattered in a pattern consistent with endothelial cell distribution. DiI-Ac-LDL can be taken-up by either endothelial cells or by macrophages however neither aortic ring nor outgrowth labelling was consistent with macrophage staining (Voyta *et al.* 1984). Further attempts to identify outgrowth cells by extracting RNA in the Matrigel mouse aortic ring model proved unsuccessful despite extensive method refinements. Reasons for failing to extract RNA from the outgrowths in the present study included the relatively low number of cells, the difficulties with dispersing Matrigel and the fact that aortic rings cultures were embedded within the Matrigel not grown on the surface. Matrigel resistance to penetration may also explain the necessity to increase the incubation time for DiI-Ac-LDL and precluded the use of standard immunohistochemistry stains to identify cells.

Growth factor requirements for the sprouting vessels were carefully evaluated in the course of method development. Conditions were optimised to produce sufficient new vessels to be able to inhibit angiogenesis with glucocorticoids but not too much growth as to make quantification inaccurate. Preliminary studies were performed in the presence of the EGM-2 bullet kit as a previous study had indicated that without it angiogenesis would be too sparse to evaluate an angiostatic compound (Rohan *et al.* 2000). Growth with the inclusion of all components was too prolific to quantify

beyond day 3, thus a process of growth factor selection was commenced to achieve optimum media conditions. Initially FBS was excluded along with hydrocortisone; in part this was to control steroid concentrations but also to encourage the selective growth of endothelial microvascular sprouts in favour of fibroblasts (Nicosia & Ottinetti 1990). Although serum free conditions did permit sufficient and quantifiable growth to contemplate the use of angiogenesis inhibitors the presence of the remaining growth factors (VEGF, FGF, IGF-1 and EGF) it concealed any putative effects of glucocorticoids.

Previous work in the rat aortic ring model and pilot studies (by Miss Katrin Burger) using VEGF immunosorbent assay in our lab (data not shown) indicated that the aortic rings generate sufficient VEGF and also FGF whilst in culture to promote angiogenesis and probably did not require exogenous addition of these factors (Villaschi & Nicosia 1993; Nicosia *et al.* 1997; Nicosia *et al.* 1994a). In addition IGF and EGF, although both acknowledged to promote angiogenesis in aortic ring models, are less potent than VEGF and FGF and in the dose ranges supplied are probably not effective (Villaschi & Nicosia 1993).

Exclusion of VEGF, FGF, IGF and EGF from the incubating media did not limit the angiogenic response and sufficient growth occurred to investigate the effects of glucocorticoids, indomethacin and LNNA. The observed inhibition of angiogenesis by glucocorticoids, concealed by the presence of exogenous growth factors but manifest in their absence, suggests an interaction between glucocorticoid and the growth factors. It is possible that exogenous addition of factors was able to overcome glucocorticoid-induced impairment of growth factor production. Further discussion regarding the possible mechanisms of glucocorticoid angiostatic effects will follow.

EGM-2 components heparin and ascorbic acid were used to supplement MCDB-131 media along with antibiotics. Heparin although not required for the angiostatic effects of high concentrations of glucocorticoids in rat aortic rings (Nicosia & Ottinetti 1990) was included in this model because of earlier reports of an as yet unexplained ability to interact with glucocorticoids to inhibit angiogenesis (Folkman *et al.* 1983; Aranyi & Machovich 1985; Sakamoto *et al.* 1987; Derbyshire *et al.* 1996; Crum *et al.* 1985). Ascorbic acid was included because of reports that this compound promotes angiogenesis in aortic ring cultures and maintains new vessel structure: possibly by influencing collagen basement membrane synthesis (Nicosia *et al.* 1991).

Choice of basement membrane matrices to be used in the studies was decided as a result of pilot studies comparing a standard and growth factor reduced (GFR) preparation of Matrigel. Previous investigators had used standard Matrigel (Rohan *et al.* 2000) but as other groups began to utilize the mouse aortic ring assay, use of GFR Matrigel appeared (Reynolds *et al.* 2002). In these latter reports using GFR Matrigel the number of vessels was smaller than in our initial studies with standard Matrigel so we compared the 2 formulations. We found, however, that angiogenesis was equally prolific using either formulation under the conditions developed in this chapter. Nevertheless as we had observed the influence of glucocorticoids using the standard preparation and as a consequence of availability problems with the GFR formulation, standard Matrigel was adopted as the matrix of choice.

Recognition of discrepancies in the number of vessels recorded in mouse aortic ring models in the literature and the work presented here also prompted a review of quantification techniques. Angiogenesis quantification has been performed by

counting new vessels using published guidelines (Nicosia & Ottinetti 1990). In fact one reason for the inconsistencies with vessel numbers in the literature and this work is in the day chosen to assay angiogenesis; other authors have found approximately 50 vessels on day 5 of culture (Rohan *et al.* 2000) which in fact is similar to the vehicle treated aortae in the current studies at that time point. Other quantification techniques were developed in the model including diameter of growth and maximum diameter of growth; these were used as a measure of the branching ability of the new vessels. Longer vessels might be expected if branching were inhibited. Since this work was completed other groups have begun to examine vessel length as a measure of branching in recognition of the importance of this process to vascular remodelling (Huang & Kontos 2002). Computer analysis techniques have been use to count vessels in the aortic ring assay although their use is not widespread (Nissanov *et al.* 1995). In this current study computer assessed vessel density was not reliable or consistent however other image analysis quantification methods corroborated the findings of vessel counting. Each technique showed a similar pattern of growth, an inhibition of growth by glucocorticoids and a similar degree of inhibition. However, in line with established techniques in the literature, vessel counting was used in these studies as the method of angiogenesis quantification. Since vessel counting was time consuming it was decided to examine vessel counts on day 7 alone as the preliminary findings indicated that angiogenesis rate slowed beyond that time point and because by day 7 the effects of angiostatic compounds (LNNA, and glucocorticoids) were significant. This was similar to but not identical to time points chosen to quantify angiogenesis in other studies (Rohan *et al.* 2000), however the reasons for the different time points is not made clear in these reports. Growth in our work even in

the absence of exogenous growth factors was more prolific than published reports (Rohan *et al.* 2000; Nicosia & Ottinetti 1990): the reasons for this are uncertain but may reflect different practices in terms of vessel preparation, size of incubating wells, and amount of Matrigel used. Great care was taken not to damage the endothelium during dissection with the use of sharp instruments and minimal vessel handling. Transfer time from tissue extraction from the animal and embedding in Matrigel was reduced with practise and may account for the increased angiogenesis seen in experiments as the technique was perfected during the course of this study.

3.4.2 Influence of glucocorticoids

Assessment of the suitability of the assay to examine the role of putative angiogenesis inhibitors was confirmed by using acknowledged angiogenesis inhibitors LNNA and indomethacin (Babaei *et al.* 1998; Dormond *et al.* 2001; Papapetropoulos *et al.* 1997). In the current study LNNA and indomethacin were used at concentrations that had previously been shown to alter vascular function (Babaei *et al.* 1998; Leyssac *et al.* 1975). LNNA inhibited angiogenesis confirming that *in vitro* NO is an important regulatory factor of new vessel formation (Lee *et al.* 1999; Zhao *et al.* 2002). In contrast at the concentration of indomethacin used angiogenesis was not inhibited. Although recognised to inhibit vessel wall production of prostanoids at the concentration used the reported anti-angiogeneis effects were noted at five times this concentration (Dormond *et al.* 2001; Jones *et al.* 1999). Other reasons for a lack of inhibition by indomethacin could be an effect of the assay conditions or as a consequence of compensation by NO. NO generation by eNOS reportedly compensates for reduced prostanoid production in endothelial cell culture however the converse effect from inhibition of NO does not occur (Vassalle

et al. 2003). The lack of angiogenesis effect from a cyclo-oxygenase inhibitor may also reflect the absence of systemic inflammation in this model and it may be that, in the presence of other facets of angiogenesis including cellular inflammatory responses, inhibition of prostaglandin and thromboxane generation would be effective.

Physiologically relevant concentrations of glucocorticoids were used to examine the relevance of glucocorticoid-induced angiostasis to physiology and pathophysiology. Angiogenesis in aortic ring cultures was inhibited by glucocorticoids at concentrations equal to and greater than 30nM with no inhibition at 3nM. It is likely even at nadir concentrations that glucocorticoids would be of the order of 30nM and thus influence angiogenesis (Harris *et al.* 2001). Although the angiostatic effects of endogenous glucocorticoids have not been examined there are examples of angiogenesis associated processes being impaired in the presence of excess endogenous glucocorticoids for example impaired wound healing in Cushing disease patients (Gordon *et al.* 1994). In addition excess production of endogenous glucocorticoids in functional adrenal cortex adenomas is associated with reduced vascularity in comparison to normal adrenal cortex (Sasano *et al.* 1998; Bernini *et al.* 2002).

The ability of 11dehydrocorticosterone to inhibit angiogenesis was also noted although no threshold concentration was determined. The concentrations of 11dehydrocorticosterone were chosen to parallel those for corticosterone. However physiological intracellular concentrations of 11dehydrocorticosterone are difficult to predict for although 11dehydrocorticosterone is present at 50nM concentrations in

plasma in mice (Kotelevtsev *et al.* 1997) the intracellular concentration may vary greatly depending on the balance of 11βHSD1 and 2 activity.

The ability of 11dehydrocorticosterone to inhibit angiogenesis raised the possibility that these compound were working either by a non-genomic mechanism, or via GR, MR or the putative 11dehydrocorticosterone receptor (Sheppard *et al.* 1998). At relevant concentrations neither mineralocorticoid receptor agonism nor antagonism influenced angiogenesis. Previously the mineralocorticoid receptor antagonist spironolactone had been found to inhibit angiogenesis; however these reports should be interpreted with caution since they were performed using supra-therapeutic concentrations and the effect was explained by inhibition of endothelial cell proliferation; without an effect on endothelial cell migration (Klauber *et al.* 1996; Gardiner *et al.* 1989; Guggino *et al.* 2003; Guggino *et al.* 2002). In the current studies similar large concentrations dramatically reduced angiogenesis and, although no direct measurements were taken of endothelial cell apoptosis or proliferation, the appearance of cultures suggested a toxic effect of spironolactone.

In contrast, the glucocorticoid and progesterone receptor antagonist RU38486 had no effect on angiogenesis consistent with previous reports (Yamamoto *et al.* 1994). Since progestogens were not added to the assay the progesterone receptor effects of RU38486 were not relevant in this model. RU38486 abolished corticosterone and 11dehydrocorticosterone-induced angiostasis and therefore glucocorticoid-induced inhibition of new vessel formation is glucocorticoid receptor dependent.

Conversion of 11dehydrocorticosterone to active glucocorticoid by 11βHSD1 was confirmed by the intact aortic ring activity assay. Dehydrogenase and reductase

activities were recorded in intact aortic rings; however, since 11βHSD2 is an exclusive dehydrogenase (Seckl & Walker 2001), the reductase activity was performed by 11βHSD1. Additional work has subsequently provided further evidence that 11βHSD1 functions in the vessel wall as a reductase enzyme since in aortae from 11βHSD1 deficient transgenic mice no measurable reductase activity was recorded.

Pharmacological inhibition of 11βHSD1 by carbenoxolone attenuated the effects of 11dehydrocorticosterone to inhibit angiogenesis but had no effect on the influence of corticosterone. Carbenoxolone at high concentration prevented any growth reflecting the reported endothelial toxicity of this compound at higher concentrations (Ullian *et al.* 1996).

The effect of transgenic deletion of 11βHSD1 on glucocorticoid-induced angiostasis was also examined. Aortic rings form mice deficient in 11βHSD1 (11βHSD1 -/-) had angiogenic responses similar to wild type aortic rings. Corticosterone inhibited angiogenesis in both 11βHSD1 -/- mice and wild types, however 11dehydrocorticosterone no longer inhibited angiogenesis in 11βHSD1 -/- mice. Thus the presence of 11βHSD1 was necessary for the angiogenic effect of 11dehydrocorticosterone. Therefore, 11βHSD1 influences the angiostatic effects of glucocorticoids through the regeneration of active glucocorticoid from inactive 11keto-compounds. In this fashion 11βHSD1 amplifies the angiostatic effects of glucocorticoids *in vitro*. The effect of 11βHSD1 required the presence of exogenous glucocorticoids and it will be important to pursue these findings *in vivo* to examine the effects of 11βHSD1 in the presence of endogenous glucocorticoids.

The molecular mechanisms of glucocorticoid-induced angiostasis are unclear but may involve glucocorticoid anti-inflammatory, anti-proliferative, anti-migratory and anti-collagen type actions. To begin to investigate a direct effect of glucocorticoids on key angiogenesis factors, physiologically relevant concentrations of glucocorticoids were used in HUVEC culture to see if the calcium responses of these cells following VEGF stimulation could be influenced. Local attempts to isolate and culture mouse aortic endothelial cells have proved unsuccessful (Christy *et al.* 2003), however a locally available surplus of human endothelial cells encouraged the use of human cells for these studies. HUVECs have previously demonstrated a bi-phasic intracellular calcium $[Ca^{2+}]i$ response to VEGF stimulation (Cunningham *et al.* 1999; Faehling *et al.* 2002; Jiang *et al.* 2001) . VEGF elicits this response through the VEGF-2 receptor (VEGFR2) (Cunningham *et al.* 1999). Although the function of other VEGF receptors in endothelial cells (for example VEGFR-1) is unclear available data suggest that the intracellular calcium responses are not induced through VEGF stimulation of this receptor (Cunningham *et al.* 1999). Inhibition of this response has been associated with impaired endothelial cell proliferation and migration (Cunningham *et al.* 1999; Faehling *et al.* 2002; Jiang *et al.* 2001). Furthermore in the course of this thesis it came to light that other endogenous angiogenesis inhibitors impaired $[Ca^{2+}]i$ responses (Jiang *et al.* 2001) . Thus the effects of pharmalogical and endogenous concentrations of glucocorticoids on endothelial cell calcium responses were studied. The results represent a pilot study and are preliminary findings. Pharmacological doses of glucocorticoid markedly attenuated the second phase of the intracellular calcium response whereas endogenous concentrations reduced the amplitude of this phase response to a lesser

degree. The biphasic response is generated from an initial reaction to liberate intracellular calcium stores through inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG). IP3 interacts with IP3 receptors on the endoplasmic reticulum to release stored calcium (McLaughlin & De Vries 2001; Faehling *et al.* 2002; Berridge 1987). The second phase is ascribed to extracellular calcium entry via storage-operated channels (SOC). The transduction system that triggers SOC opening is still unclear but is thought to be effected by increases in $[Ca^{2+}]i$, DAG, IP3, or all three (Berridge 1987; Nilius & Droogmans 2001).

Glucocorticoids appear to influence the entry of extracellular calcium ions. The channels activated by VEGF are believed to be different to those activated by other endothelial vasomotor agonists (for instance ATP) (Pocock *et al.* 2004). In fact the SOC are a diverse group of channels and are encoded by the gene family transient receptor potential 1-7 (TRP1-7). TRP 1-6 are expressed in mammalian endothelial cells (Nilius & Droogmans 2001). There is evidence for the involvement of these channels in VEGF responses: TRP-6 inhibition does attenuate VEGF stimulated fluctuations in $[Ca^{2+}]i$ (Pocock *et al.* 2004) however its presence in HUVECs remains undetermined. TRP1 and 3 are expressed in HUVECs, however the absence of a specific antagonist has limited further investigation of their roles and their role in VEGF responses has not been examined. A further candidate for glucocorticoid action may be TRP-4: endothelial cells from TRP-4 -/- mice lack a store dependent calcium current and exhibit intracellular calcium responses similar to those seen in cortisol exposed HUVECs (Plant & Schaefer 2003). TRP proteins are activated probably by several means including by IP3, DAG by intracellular calcium or via receptor generated phosphorylation pathways. It is unclear which of these pathways

glucocorticoids might interrupt and this was beyond the scope of this thesis although the experimental findings are being followed up through the work of a new PhD student.

Further studies with the models presented in this chapter are planned for a new project with the aim to elucidate some of the mechanisms to explain the results. The aortic ring assay will be used to assay glucocorticoid interactions with key angiogenesis factors including NO, VEGF and FGF. Identification of NO metabolites in aortic ring culture medium by chromatography and FGF and VEGF by ELISA studies are being considered. The aortic ring assay has also been use in a hypoxic chamber that became available during the course of these studies. Controversy regarding the effects of glucocorticoid on angiogenesis factors in hypoxic conditions exists (Kodama *et al.* 2003) and this chamber will allow experimentation to investigate some of these controversies *in vitro*. Furthermore hypoxic studies of angiogenesis are also useful to examine the role of endogenous glucocorticoids in pathophysiology, for example in tumours and in infarcted tissue. The hypoxic environment of such pathologies will produce different angiogenesis conditions and the effect of glucocorticoids to regulate angiogenesis in these conditions will need to be assayed.

Extraction of RNA from aortic rings in Matrigel has proved difficult. This may reflect the Matrigel or the small amount of cellular material. Plans have been discussed to trial the collagen gels or learn the techniques performed by Nicosia et al (Nicosia *et al.* 1994b) that allow them to isolate the cells grown from the aortic rings. This will allow us to elucidate the expression of 11βHSD-1 and examine the expression of key angiogenesis factor e.g. VEGF, eNOS to see if glucocorticoids

influence these. Finally following criticisms of the aortic ring model because of apparent controversies regarding the nature of the outgrowths attempts are being considered to perform electron microscopy (Nicosia & Ottinetti 1990) to establish whether the outgrowths are endothelial lined luminised tubes.

The data in this chapter provide evidence for the establishment of a novel *in vitro* assay to examine the effects of putative angiostatic factors and examine the effects of physiologically relevant concentrations of glucocorticoids on angiogenesis. Glucocorticoids at these concentrations inhibited angiogenesis in a glucocorticoid receptor dependent manner. Furthermore, the inert 11ketocompound 11dehydrocorticosterone, a substrate for 11βHSD1, also inhibited angiogenesis in a glucocorticoid receptor dependent manner. The effect of 11dehydrocorticosterone was attenuated by pharmacological inhibition or gene deletion of 11βHSD1. Glucocorticoids at physiological relevant concentrations inhibited the intracellular calcium concentrations of endothelial cells in responses to VEGF. Thus physiological concentrations are sufficient to inhibit angiogenesis and are modulated by the effects of vessel wall 11βHSD1. It is important to verify these findings *in vivo* to confirm whether 11βHSD1 influences the effects of endogenous glucocorticoids to regulate angiogenesis, and to examine whether this regulatory mechanism has a role in controlling angiogenesis in pathology.

Chapter 4

11β Hydroxysteroid Dehydrogenase type 1 Influences Regulation of Angiogenesis by Endogenous Glucocorticoids *in vivo*

4.1 Introduction

Whilst physiologically relevant concentrations of steroids inhibited new vessel formation *in vitro* it remained unclear whether endogenous glucocorticoids influence angiogenesis *in vivo*. Previously, compounds that have inhibited endothelial cell migration or proliferation or differentiation *in vitro* have not necessarily regulated endothelial cell activity *in vivo* (Liekens *et al.* 2001). Therefore, although novel, the *in vitro* results described in Chapter 3 required further justification *in vivo* in order to assess their physiological relevance.

In vivo models of angiogenesis differ from *in vitro* assays by incorporating systemic factors, including: inflammation, hormones, metabolic homeostasis and circulating vascular-precursor cells. These different conditions found *in vivo* are particularly pertinent to the studies described in this thesis since glucocorticoids influence inflammation (Barnes 1998), are hormones that act at distant sites, they regulate tissue metabolism (Wake & Walker 2004), alter cell differentiation(Amanatullah *et al.* 2002) and mobilisation of progenitor-cells (Mao *et al.* 2004). Thus the development of a suitable *in vivo* model was essential to confirm the influence of endogenous glucocorticoids on angiogenesis.

There are several *in vivo* models applicable to rodent studies that might be relevant, such as the corneal model and chamber or dorsal sac assays (Staton *et al.* 2004). These, however, are expensive, technically demanding and in the main utilise cancer cells. As the study of angiogenesis in tumours was not the focus of this thesis, these techniques were not pursued. Instead a subcutaneous implant assay was adopted. There was a choice of implant assays as both Matrigel and sponge models are established in the literature (Andrade *et al.* 1987; Biancone *et al.* 1997; Hague *et al.* 2002). Whilst Matrigel is expensive and quantification of implanted gels is

technically demanding, costly and inconsistent (Staton *et al.* 2004); polyester sponge implantation used in this chapter, is simple to perform, relatively inexpensive and angiogenesis is quantified by using simple histology. Refinements of the sponge implantation assay were necessary because of technical difficulties associated with the manner of drug administration (Hague *et al.* 2002; Hori *et al.* 1996). This model was adapted to reduce animal stressors by developing a silicon pellet impregnated with treatment that could be placed into the centre of the sponges. The importance of reducing animal stress was to examine the influence of physiological, rather than stressed, concentrations of glucocorticoids on angiogenic responses and determine the receptor responsible for these effects. For, although therapeutic concentrations of glucocorticoids inhibit angiogenesis (Hori *et al.* 1996), it is unclear whether this is a glucocorticoid or mineralocorticoid receptor mediated effect. Although by using pellets it was possible to administer steroids, the final concentrations of glucocorticoids within an implant would be determined by 11βHSD metabolism. *In vitro* 11βHSD1 influenced the angiostatic effects of glucocorticoids, however, a similar role for this isozyme *in vivo* had not been shown. Transgenic 11βHSD1 deficient mice are ideal models to investigate the influence of 11βHSD1 on angiogenesis *in vivo*. Although these animals have no developmental vascular abnormalities (Kotelevtsev *et al.* 1997; Hadoke *et al.* 2001) it is not unusual for angiogenesis factor gene deletions to be associated with normal adult vasculature (Carmeliet & Collen 2000). In some cases the importance of factors only become noticeable upon tissues stress either following injury, infarction, or tumour implantation.

Therefore the aims of this study were to establish an *in vivo* assay of angiogenesis to examine the influence of endogenous glucocorticoids to modulate angiogenesis. In addition the model was used to identify the receptor responsible for glucocorticoid-mediated angiostasis and investigate the influence of 11βHSD1 on angiogenesis *in vivo*.

4.2 Methods

Experimental procedures were adopted and refined from previous models (Hori *et al.* 1996; Hague *et al.* 2002) to establish a more suitable *in vivo* assay to examine the effect of endogenous glucocorticoids on angiogenesis. Implantation technique, drug delivery, experiment duration, methods for quantification of steroids within the sponge and quantification of angiogenesis all underwent extensive development as detailed in the following sections.

4.2.1 Development of the subcutaneous sponge implant assay

(Please refer to section 2.7)

Mice- Male 10-12 week old, C57Bl6 and homozygous null 11β-HSD 1 (bred to be congenic) male mice were used.

Sponges- White, grade XE1700V polyester sponge was used (Caligen Foam Limited, UK). Other sponge samples were sourced locally (Didcock Bros, UK) but proved unsuitable because of differences in porosity, colouring, grade and thickness.

Implantation procedure- (See section 2.7.3) Training in sponge implantation techniques was kindly provided by Mrs Sandra Peak at the Imperial Cancer Research

Fund Clare Hall Laboratories (Potters Bar, UK). The anaesthetic and implantation techniques learnt at training were initially employed with minor alterations: animals received an intraperitoneal anaesthetic fentanyl/ fluansone and midazolam (as Hypnorm (Janessen, UK) /Hypnovel (Roche, UK) 0.1ml/ 10g of a solution containing Hypnorm 1.0 ml + Hypnovel 1.0ml + sterile water 2.0 ml). This was used instead of intraperitoneal tribromoethanol (Avertin) that had been administered at Clare Hall to avoid reported peritoneal adhesions, liver damage and post operative mortality (Zeller *et al.* 1998). Anaesthetized mice underwent subcutaneous sponge implantation with each mouse receiving 2 sponges; one on each flank. This was an adaptation of established procedures using mice in this assay (Hague *et al.* 2002; Illanes *et al.* 2002) but similar to the original descriptions in rats (Hori *et al.* 1996). With practice, the need for intraperitoneal anaesthesia with a long half-life was reduced and Halothane inhalation anaesthesia was adopted as standard procedure.

4.2.2 Administration of steroids

Several different routes of drug delivery have been used with subcutaneous sponge implants (Hori *et al.* 1996; Hague *et al.* 2002; Illanes *et al.* 2002). In the current study a silastic pellet containing steroid or vehicle was used, these pellets could release steroid at a controlled rate for 3 weeks (Cleasby *et al.* 2003) and could be placed adjacent to or within implanted sponges promoting localised delivery and reduction in handling and housing stresses.

Implant manufacture- (See section 2.7.1) Steroid was added to Silastic elastomer (Dow Corning Corporation) mixed and cured overnight at 25^0C either in the 1ml syringe or following injection into capillary tubing (1.2mm-0.76mm) (Portex Ltd,

UK). Placebo, control pellets were manufactured in the same manner without the addition of steroid. Following curing, capillary tubing was split and pellets cut into 1cm pieces weighing 10-12mg, the 1ml syringe was cracked open and the pellet cut into 0.5cm pieces weighing 50mg each.

Drug Administration- Initial studies using pellets containing cortisol or placebo, were performed by placing the pellet adjacent to the sponge in the subcutaneous pocket. Curing of pellets within capillary tubing enabled the manufacture of thin tubes of silicon containing steroids that could be inserted into the centre of the sponge cylinder. This technique was adopted as standard procedure for the remainder of the experiments (See section 2.7.1). Drug dosages were calculated following preliminary experiments in which a high dose of corticosterone (25mg) had been used. Large concentrations of corticosterone were associated with systemic side effects. Steroid dosages were therefore titrated to reduce the incidence of systemic side effects yet influence new vessel formation.

Experiment duration- Studies in rats indicated that the subcutaneous tissues underwent a predictable response to sponge implantation (Andrade *et al.* 1987). Angiogenic responses in these studies and in mouse models of the assay (Illanes *et al.* 2002; Hague *et al.* 2002) were measured 3 weeks after sponge implantation and thus this time point was used in the current studies.

Assessment of the systemic effects of administered agents- Subcutaneous wounds were regularly inspected to examine hair growth, wound granulation and wound

apposition. In addition, animals were weighed daily and their water intake measured to monitor their welfare and assess the systemic effects of administered drugs.

Tissue collection- (See section 2.8. 3 weeks following implantation (Hori *et al.* 1996), mice were decapitated, trunk blood collected into lithium heparin tubes, sponges and organs excised and inserts removed. Sponges were bisected; one half was fixed in 10% formalin, embedded in paraffin wax and cut using a microtome (Hague *et al.* 2002). Sections (8μm) were stained with haematoxylin and eosin (H&E) for quantification of angiogenesis.

To determine the concentration of steroid or protein in sponges, the remaining half of the sponge was weighed and placed in ice-cold phosphate buffer solution (PBS) and homogenised. The homogenate was centrifuged, 40μl of the supernatant were used to determine sponge protein colormetrically using the Bradford assay (Bradford 1976) (See section 2.7.6). A further 750μl of the supernatant were removed, added to 7.5 ml of ethyl acetate vortexed and allowed to settle. 7ml from the viscous phase of this mixture were removed, dried and then assayed for steroid concentration.

*Steroid Quantification-*The concentration of steroid in the sponges and mouse plasma was determined by radioimmunoassay (See section 2.8.5). However, as anti-cortisone anti-bodies (used in the radio-immuno assay to detect cortisone) cross-react with cortisol a further purification step was required (Table 4.1(Lloyd-MacGilp *et al.* 1999)). Cortisone and cortisol in dried samples were separated using high-performance liquid chromatography (HPLC) (Walker *et al.* 1991) (see section 2.8.8).

Separation of cortisol and cortisone in the chromatography column allowed separate collection of each steroid at a time point defined by control standards.

Ultra violet light detection was used for samples from 11βHSD-1 -/- mice to determine the absence of cortisol in cortisone treated sponges. As no cortisol was detected in 11βHSD-1 -/- mice that had received cortisone (Figure 4.11) the HPLC step in quantifying steroids from these animals was discontinued.

4.2.3 Quantification of angiogenesis in sponges

In preliminary experiments both histological and immunohistochemical techniques were tested to determine the best method for identifying blood vessels within the sponge matrix. Attempts to stain endothelial cells with either anti-CD31 (BD Biosciences, UK) or anti-vWF (Dakocytomation, UK) antibodies proved, despite extensive adjustment of the staining protocols, to be unsuccessful. In contrast, blood vessels were readily visible in sections stained with haematoxylin and eosin (H & E). In preliminary reports of the sponge implant model (Andrade *et al.* 1987; Edwards *et al.* 1960) vessels had been identified using simple haematoxylin and eosin staining and following discussions with a leading angiogenesis investigator this method was adopted. Initially, quantification was performed using 2 different counting protocols: firstly, 3 high power fields (x100) from 2 sections of same sponge were assessed and the mean calculated. Following this, vessel density determination by Chalkley counting (Fox *et al.* 1995) was measured. The mean of triplicate Chalkley counts on 2 sections per sponge was used. Comparison of the 2 vessel counting techniques demonstrated that both were reliable, reproducible and produced similar findings. As

Chalkley counting is more time efficient it was adopted as standard procedure (See section 2.8.4) (Figure 4.1).

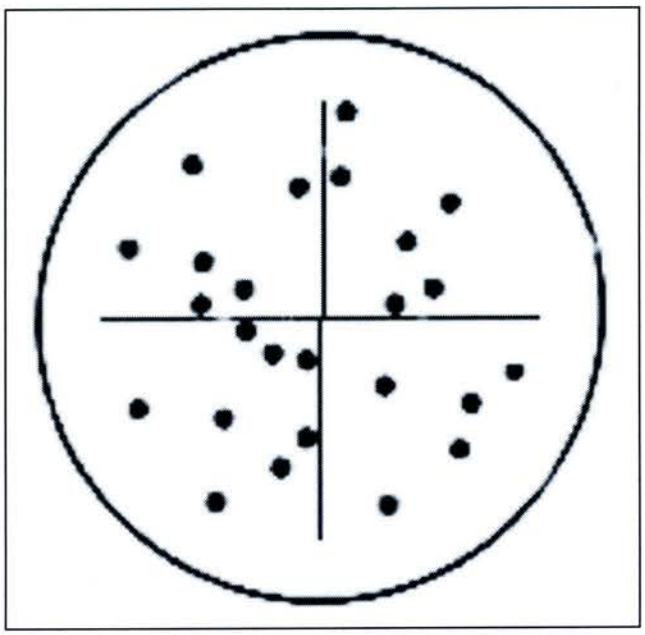


Figure 4.1 Chalkley Reticule

Chalkley counting at high power (x250) (Fox *et al.* 1995). Chalkley counting is performed using a 25-point Chalkley eyepiece reticule (Graticules Ltd, Kent UK). At low power the 3 most vascular areas were identified and analyzed using the graticule at 250x magnification. The graticule was rotated until the highest number of Chalkley reticule points were aligned with vessels and a score out of 25 was achieved. From each section three Chalkley scores were recorded thus a total of six Chalkley scores were obtained for each sponge and the mean of these was used as the Chalkley count.

	Cortisol	Cortisone	Corticosterone
Cortisol RIA	100%	0.1%	0.2%
Corticosterone RIA	10%	0.8%	100%
Cortisone RIA	6%	100%	0.5%

Table 4.1 Cross reaction of cortisol, cortisone and corticosterone in radio immunoassays

Cortisol present with samples may bind to cortisol antibodies in the cortisol RIA. In addition cortisol may also bind to corticosterone or cortisone antibodies in RIAs for these other steroids. Thus, samples from mice that received cortisol or cortisone underwent high pressure liquid purification so that the cortisol could be separated and removed (RIA radio immunoassay) (Lloyd-MacGilp *et al.* 1999).

4.2.4 Application of the assay: examining the angiostatic effects of endogenous glucocorticoids and the influence of 11βHSD-1

Validation of control sponge conditions – Control sponges were placebo-treated sponges that had been implanted in the opposite flank from a treatment sponge. To validate this novel approach, angiogenesis in the placebo treated sponge was also compared to new vessel formation in a sponge from an animal that had received no exogenous steroid (Figure 4.2).

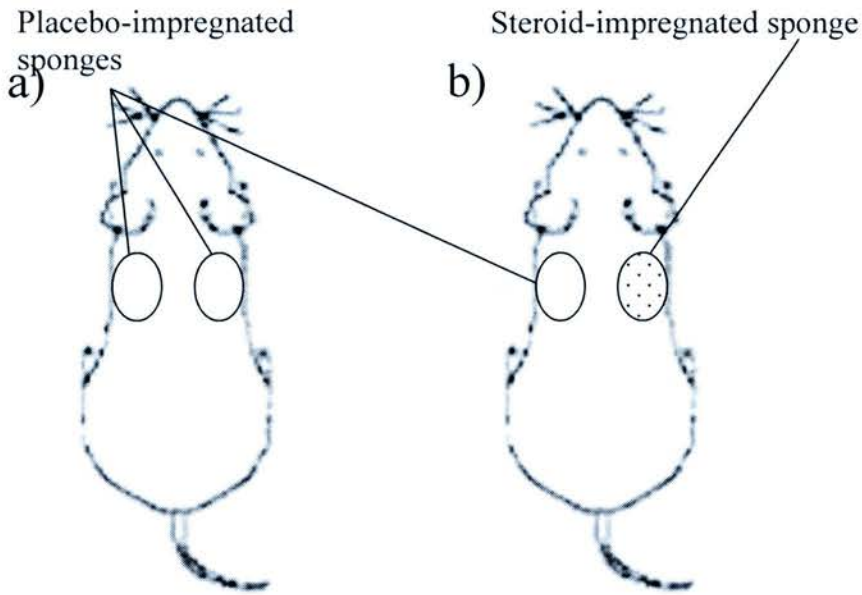


Figure 4.2 Method development: Validation of control sponges in mice that had received steroid

Control sponges were placebo-treated sponges that had been implanted in the opposite flank from a steroid-impregnated sponge. To validate this novel approach, angiogenesis in placebo-impregnated sponges (a) was compared with new vessel formation in placebo-impregnated sponge in animal that had also received steroid (b). The steroid used for this comparison was a high concentration of corticosterone (25mg).

Effect of exogenous glucocorticoids- (See section 2.8) To determine the influence of glucocorticoids on angiogenesis *in vivo,* C57Bl6 male mice received a sponge impregnated with a steroid pellet on the right flank and a placebo impregnated sponge implanted on the left flank. Steroids used included 25mg of corticosterone, 2.5mg of cortisol or 2.5mg of cortisone. Following the initial experiments with high concentrations of corticosterone, smaller concentrations of human equivalents to corticosterone (cortisol) and 11dehydrocorticosterone (cortisone) were used. Three weeks following implantation, mice were decapitated, trunk blood collected, sponges and organs excised and processed, as described (section 2.8).

Influence of endogenous glucocorticoids- (See section 2.8.2) The influence of endogenous glucocorticoids was examined using adrenalectomy or pharmacological antagonism. C57Bl6 mice underwent bilateral adrenalectomy or sham procedure (Livingstone *et al.* 2000a) followed by bilateral sponge implantation under the same anaesthetic. Dr Patrick Hadoke performed the surgery in these animals.

Antagonism of endogenous glucocorticoid action was examined using RU38486 or spironolactone. C57Bl6 male mice received bilateral sponge implants: a sponge impregnated with 5.25mg of RU38486 or 5.25mg of spironolactone was implanted on the right flank whereas the left flank received a placebo-impregnated sponge.

Moderation of the influence of endogenous glucocorticoids by 11βHSD-1- C57Bl6 and 11βHSD-1 -/- male mice received bilateral sponge implants: a sponge

impregnated with cortisol (2.5mg) or cortisone (2.5mg) was implanted on the right flank whereas the left flank received a placebo-impregnated sponge. Three weeks following implantation sponges were collected and processed.

4.2.5 Statistics

Data are mean ± SEM. Comparisons were made by ANOVA with least squares difference post hoc tests.

4.3 Results

4.3.1 Method Development

Pilot studies using high dose corticosterone pellets assessed several features of the *in vivo* assay. The results from these preliminary investigations confirmed the suitability of the model to examine the influence of glucocorticoids on angiogenesis and are detailed below.

Validation of control sponges (Figure 4.2)- Angiogenesis in placebo-impregnated sponges implanted into animals that received exogenous glucocorticoids (corticosterone 25mg via a steroid-impregnated sponge on the opposite flank) was not different (p=0.36) from angiogenesis in placebo-impregnated sponges from mice that received no steroid treatment (Figure 4.3).

Localised drug delivery- Quantification of corticosterone concentration in corticosterone-impregnated sponges compared to the contralateral placebo impregnated sponge demonstrated that steroid treatment was localised. In addition there was no evidence of cross-contamination with steroid to alter angiogenesis conditions for the sponge on the opposite flank (Figure 4.4).

Quantification of angiogenesis- Quantification of angiogenesis by the Chalkley method was compared to high power field (x100) vessel counting in preliminary studies to investigate the effects of glucocorticoids on angiogenesis. Using either technique corticosterone inhibited angiogenesis. In addition the degree of inhibition was similar for both techniques. However the Chalkley count was better established (Hague *et al.* 2002) and more time efficient and therefore was adopted as the standard method (Figure 4.5)

Figure 4.3 The influence of contra-lateral steroid implantation on angiogenesis in control sponges

Comparison of angiogenesis in placebo impregnated sponges from mice subjected to implantation of a sponge containing either a steroid (solid bar) or placebo (no steroid; open bar) insert on the contralateral flank. The number of vessels in placebo sponges was not affected by the presence of a contralateral implant containing 25mg corticosterone ($p=0.36$ $n=4$-6). Therefore placebo-impregnated sponges contralateral to steroid-sponges could be used as controls.

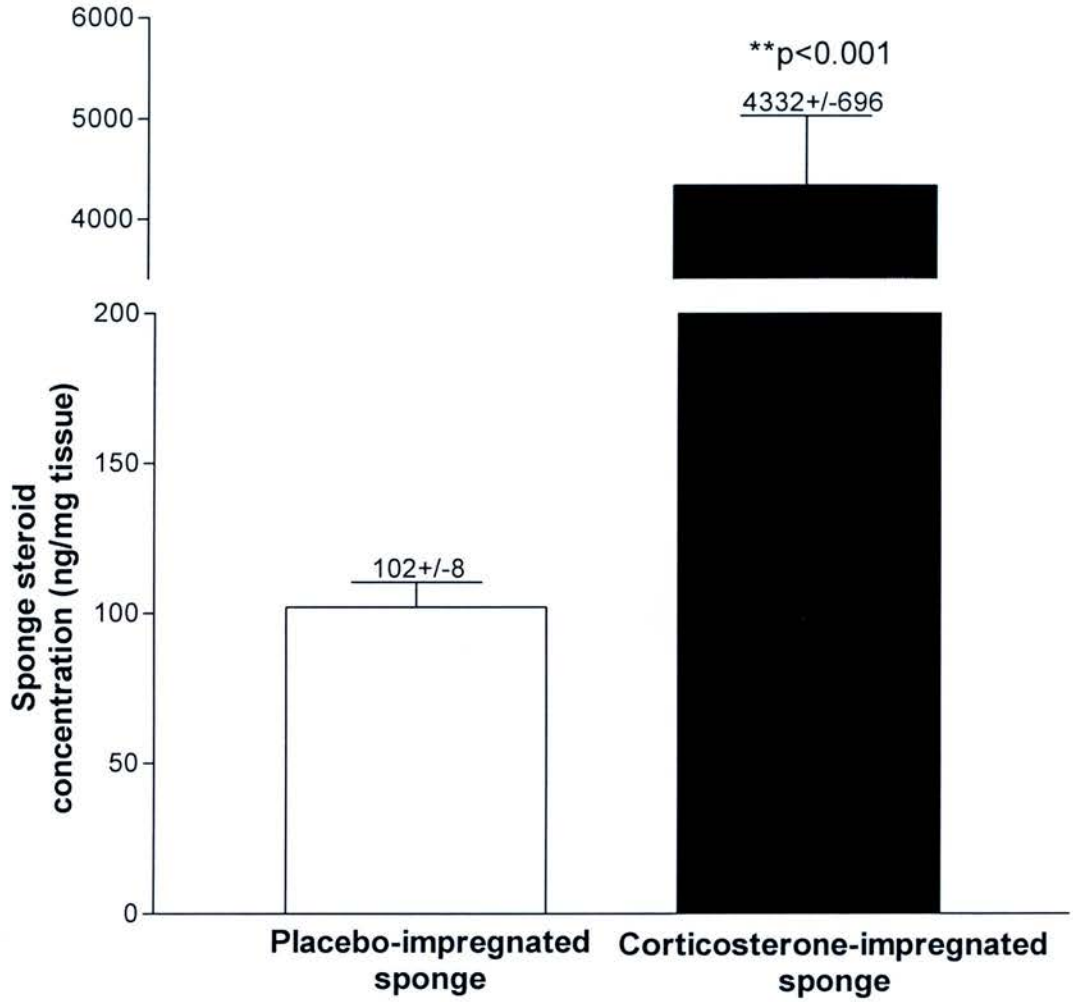
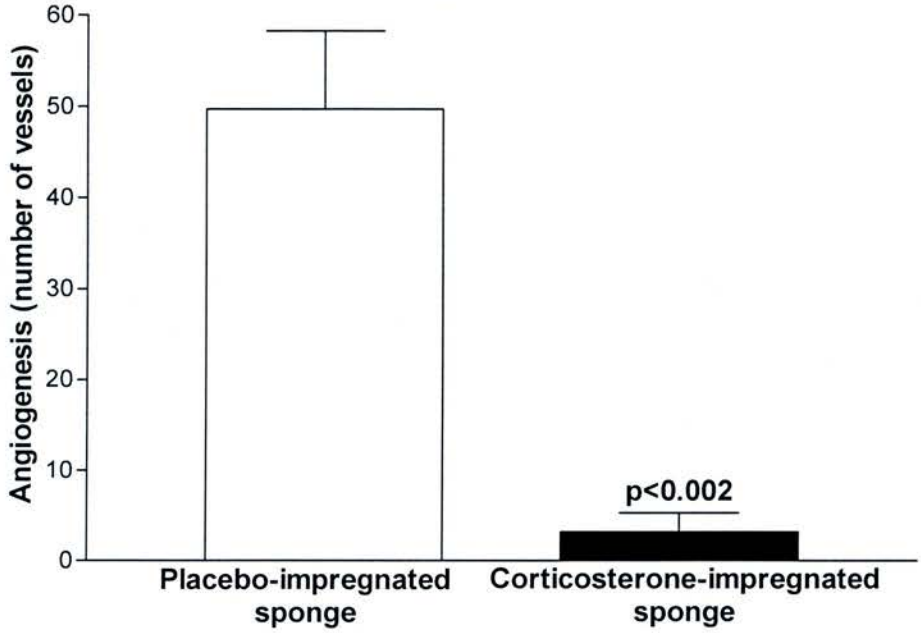


Figure 4.4 Localized drug delivery: corticosterone concentration within sponges

Corticosterone concentrations were markedly (**p<0.001) elevated in sponges containing corticosterone implants (solid bar), compared with contralateral placebo controls (open bar). Data are mean ± SEM. Comparisons were made by ANOVA (n=4).

a) Glucocorticoid-induced angiostasis assessed by vessel counting



b) Glucocorticoid-induced angiostasis assessed by the Chalkley method

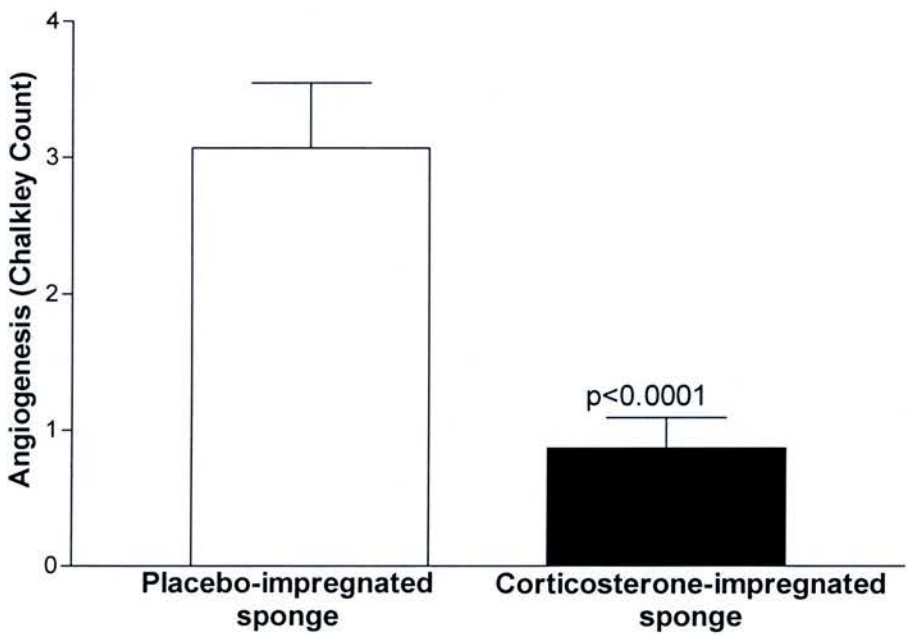


Figure 4.5 Comparing quantification techniques

Angiogenesis in sponges impregnated with corticosterone (25mg) was compared to new vessel formation in sponges impregnated with placebo using a) high power (x100) field vessel counting and b) the Chalkley method. Using either technique corticosterone was noted to inhibit angiogenesis. The degree of inhibition was similar in both methods. Data are mean ± SEM. Comparisons were made by ANOVA n=4-6.

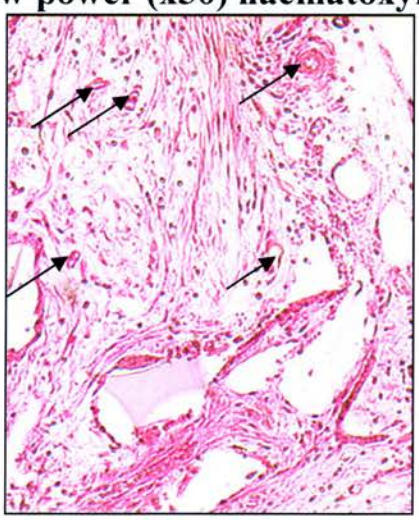
Drug dosing- Calculation of appropriate drug dosages was performed following observations made using a high concentration of corticosterone. Initially 25mg corticosterone pellets were chosen for similar pellets had been seen to be effective in delivering glucocorticoids over 4 weeks (Cleasby *et al.* 2003) and furthermore it was anticipated that, by using a high concentration, an angiostatic effect would be observed (i.e. proof of principle). With this dose of steroid there was a dramatic effect on sponge appearance (Figure 4.6). Three weeks following implantation, corticosterone-treated sponges were white with few, if any, vascular structures visible on gross examination. In contrast the contralateral placebo-treated sponges upon excision were pink with numerous vascular structures visible (Figure 4.5). These findings were confirmed at low power histology (Figure 4.6) and following quantification of angiogenesis (Figure 4.5). At this concentration corticosterone also had systemic effects: it reduced the weight of glucocorticoid sensitive organs, suppressed plasma levels of corticosterone as previously reported (Cleasby *et al.* 2003) (Figure 4.7) and influenced hair regrowth over subcutaneous incision wounds (not formally quantified).

In further studies, reduced doses of exogenous glucocorticoids, cortisol and cortisone, were used. The human equivalents of rodent glucocorticoids were employed to distinguish these steroids from endogenous glucocorticoids. 2mg pellets of cortisol and cortisone did not affect glucocorticoid-sensitive organ weights (figure 4.7a) or wound healing, did not alter plasma corticosterone (Figure 4.7b) but did inhibit angiogenesis to a similar degree to corticosterone both on gross examination and microscopic evaluation (Figure 4.8a-d).

a) Gross appearance of placebo or corticosterone treated sponges

b) Low power (x50) haematoxylin and eosin stained sections

i)



ii)

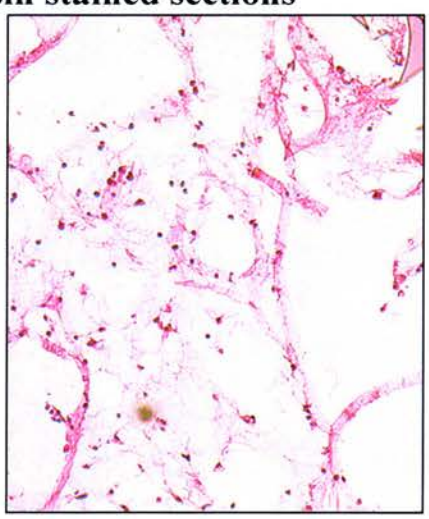


Figure 4.6(a-b) Assessing drug concentrations: corticosterone is angiostatic

Corticosterone (25mg) inhibited angiogenesis in sponges implanted subcutaneously in mice. On gross inspection (a) corticosterone sponges were avascular and white in appearance in contrast placebo treated sponges were pink and appeared to be highly vascular. At low power magnification (b) these findings were confirmed, numerous vessels were seen (black arrows) in placebo treated sponges (bi) in contrast scattered if any vessels were seen in corticosterone-impregnated sponges (bii).

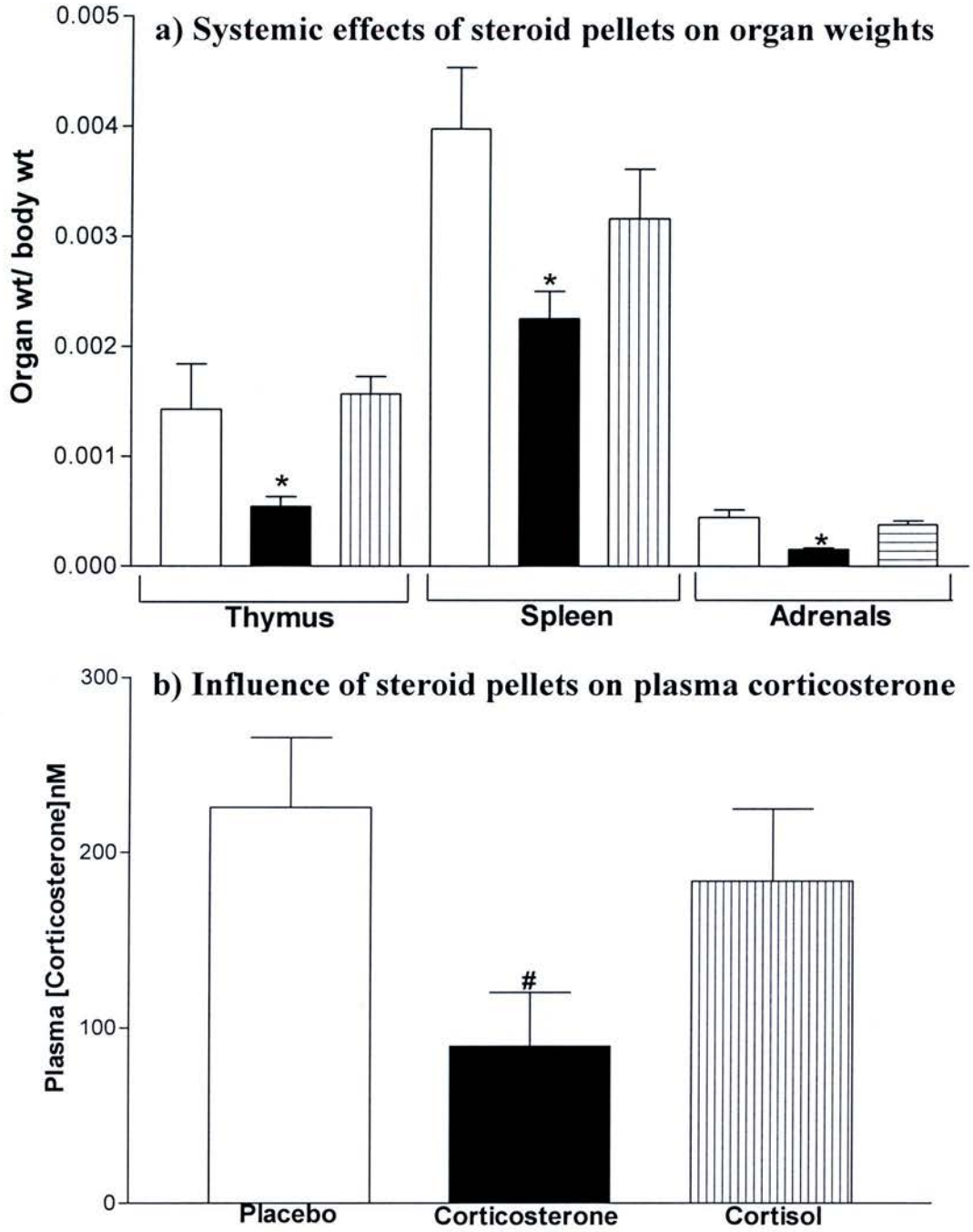
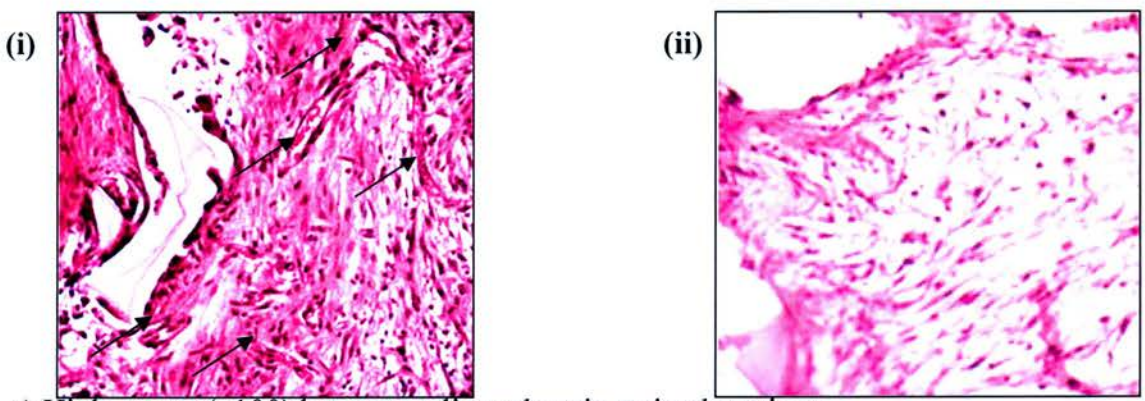


Figure 4.7 Assessing drug concentration: systemic effects of corticosterone

Systemic effects of using different concentrations of corticosterone (25mg) or cortisol (2mg) impregnated sponges, Pellets containing corticosterone 25 mg (solid bar) had systemic effects on glucocorticoid-sensitive organ weights (a) and plasma corticosterone (b) versus animals who received no steroid (clear bars), or cortisol (2.5mg) (vertical lined bars). Data are mean ± SEM *p≤ 0.04, #p<0.01. Comparisons were made by ANOVA with least squares difference post hoc tests, n=4-9.

a) Gross appearance of placebo (i) and cortisol (ii) impregnated sponges

b) Low power (x50) haematoxylin and eosin stained sections



c) High power (x100) haematoxylin and eosin stained sections

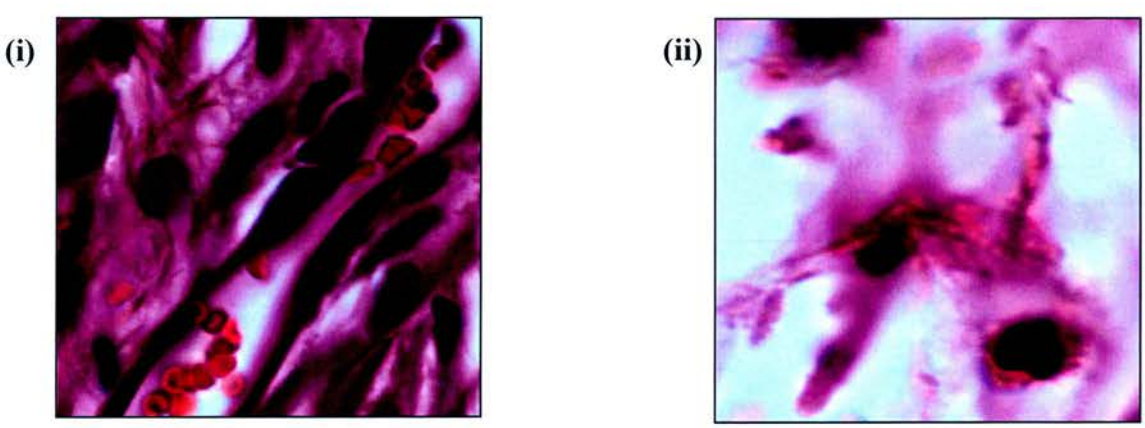


Figure 4.8(a-c) Glucocorticoids inhibit angiogenesis in vivo

Images from placebo (i) and cortisol (2.5mg) (ii) impregnated sponges. Placebo treated sponges appeared vascular on gross inspection (a), many vessels were seen at low magnification (b) at high magnification mature vessels were seen. In contrast cortisol impregnated sponges were white on gross inspection (a), few if any vessels were seen at low magnification (b) however at high magnification an inflammatory infiltrate persisted (c),

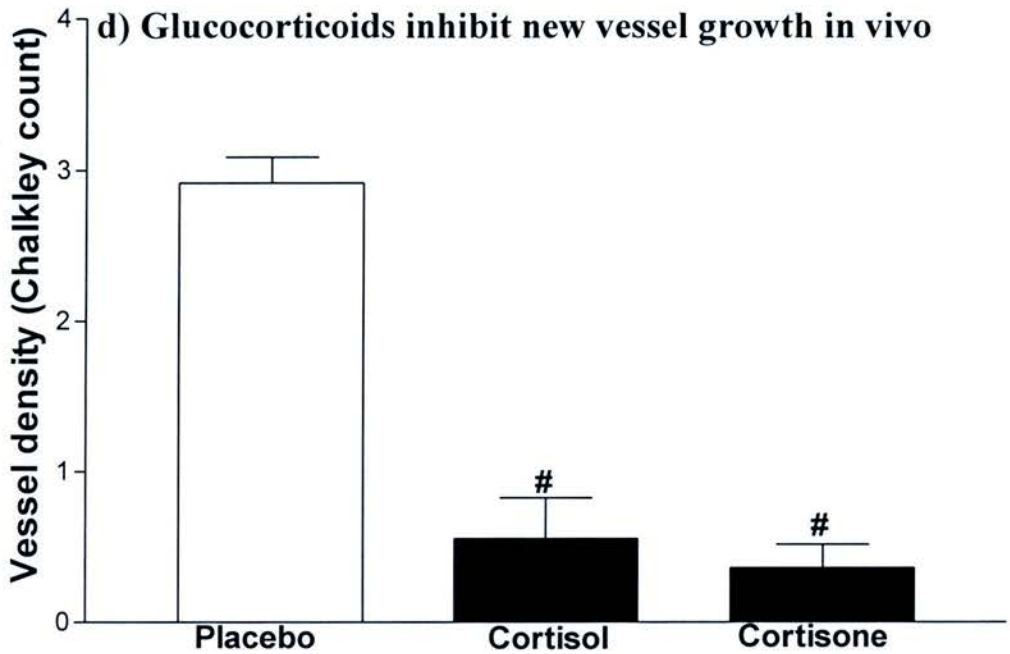


Figure 4.8d Glucocorticoid inhibit angiogenesis *in vivo*

In comparison to placebo impregnated contra lateral controls, sponges impregnated with glucocorticoids (2.5mg cortisol or cortisone) inhibited angiogenesis quantified by Chalkley counting. Data are mean ± SEM #p<0.01. Comparisons were made by ANOVA with least squares difference post hoc tests, n=6.

Influence of glucocorticoids on protein concentrations within sponges - Protein concentrations were not different in corticosterone, cortisol or cortisone impregnated sponges compared to contralateral placebo controls (Figure 4.9). The absence of an effect on sponge protein concentration, despite the evident effect on angiogenesis, indicated that this was an unreliable method of assessing the angiostatic effects of glucocorticoids and therefore was discontinued.

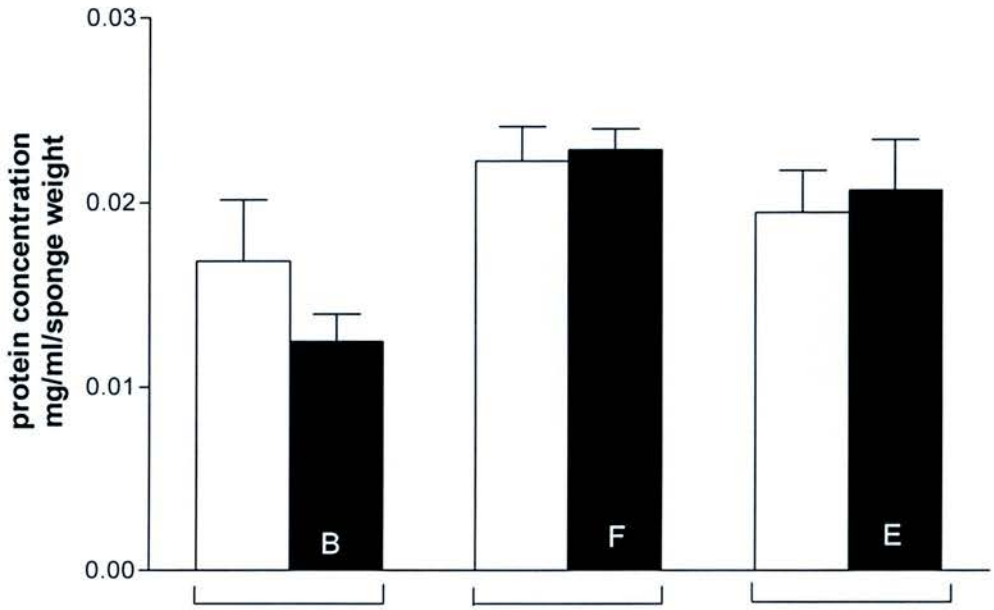


Figure 4.9 Sponge protein concentrations

Impregnation of sponges with glucocorticoids did not affect sponge protein concentration in comparison to relevant control sponges. Solid bars represent steroid impregnated sponges (B is corticosterone 25mg; F is cortisol 2.5mg, E is cortisone 2.5mg n=6). Bracketed clear bars represent contra lateral placebo impregnated controls. Data are mean ± SEM n=4-6 $p>0.23$ for comparisons with relevant controls, comparisons were made by ANOVA with least squares difference post hoc tests.

4.3.2 Moderation of the influence of endogenous glucocorticoids by 11βHSD-1

Endogenous glucocorticoids repress angiogenesis- The absence of adrenal glands at post-mortem examination and reduced plasma corticosterone concentrations (156.1±25.1nM in sham versus 46.7±6.5nM (below level of detection for this assay which is 50nM (Cleasby *et al.* 2003)) for adrenalectomised animals n=5, $p<0.001$ by ANOVA) confirmed the success of adrenalectomy. The number of blood vessels in sponges from mice that had undergone adrenalectomy was increased in comparison to sham-operated controls (Figure 4.10a).

Sponges impregnated with the glucocorticoid receptor antagonist RU38486 had increased angiogenesis in comparison to placebo-impregnated controls (Figure 4.10b). An assessment of the systemic effects of glucocorticoid receptor antagonism indicated that RU38486 reduced plasma corticosterone (93.5±32.3nM in RU38486 versus 225.8 ± 39.9nM in controls, n=6, $p<0.01$ by ANOVA) but did not alter the weight of glucocorticoid-sensitive organs (data not shown).

Spironolactone had no effect on angiogenesis in sponges (3.94±0.55 Chalkley count in controls versus 3.78±0.24 Chalkley count in spironolactone treated sponges, n=3).

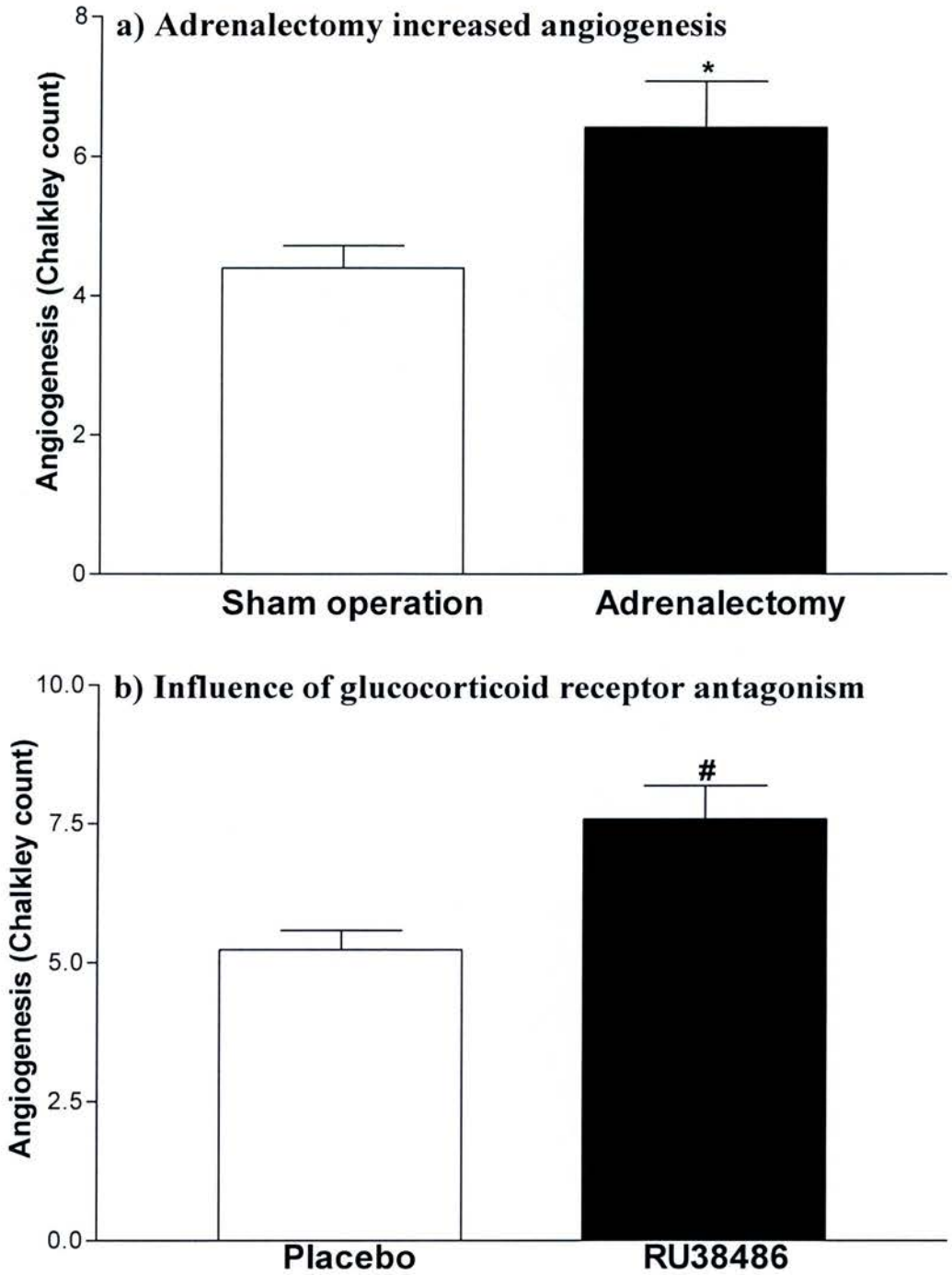


Figure 4.10 Endogenous glucocorticoids repress angiogenesis

(a) Sponges implanted into mice post-adrenalectomy (solid bar) were more vascular than control sponges implanted post sham operation (clear bar). The angiostatic influence of endogenous glucocorticoids is glucocorticoid receptor-dependent (b): sponges impregnated with RU38486 (solid bar) were more vascular than contra lateral placebo impregnated controls (clear bar). Data are mean ± SEM #$p<0.01$ and *$p=0.02$. Comparisons were made by ANOVA, n=6.

11βHSD1 tonically represses angiogenesis in vivo –To determine 11βHSD reductase activity in implanted sponges, radioimmunoassay of steroid fractions separated by HPLC was performed. This confirmed 11βHSD reductase metabolism in cortisone-impregnated sponges from C57Bl6 mice by detection of cortisol (Table 4.2). No reductase activity however was seen in cortisone-impregnated sponges from 11βHSD1 -/- mice (Figure 4.11 and Table 4.2).

Sponges impregnated with cortisol or cortisone and implanted in C57/Bl6 mice had impaired angiogenesis in comparison to placebo-impregnated sponges (Figure 4.12). In 11βHSD1-/- mice new vessel formation was inhibited in cortisol-impregnated sponges, whereas angiogenesis was not affected in cortisone-treated sponges (Figure 4.12). In addition angiogenesis was greater in placebo-sponges from 11βHSD1-/- mice compared to wild types (Figure 4.12).

4.3.3 Inter and intra-assay variation

Inter-assay variation was calculated as the variation in angiogenesis between mice. It was assessed in mice with 2 sponges containing placebo by measuring the coefficient of variation between Chalkley counts for each mouse and finding the mean of these values which was 8± 3% (n= 6). Intra-assay coefficient of variation was quantified as the variation in angiogenesis between sponges in the same animal. It was calculated in mice with 2 sponges containing placebo by measuring the variation between Chalkley counts of each sponge in the same animal and finding the mean of these from n= 6 mice and it was 12 ± 3%.

Figure 4.11 Absence of 11βHSD reductase activity in 11βHSD 1-/- mice

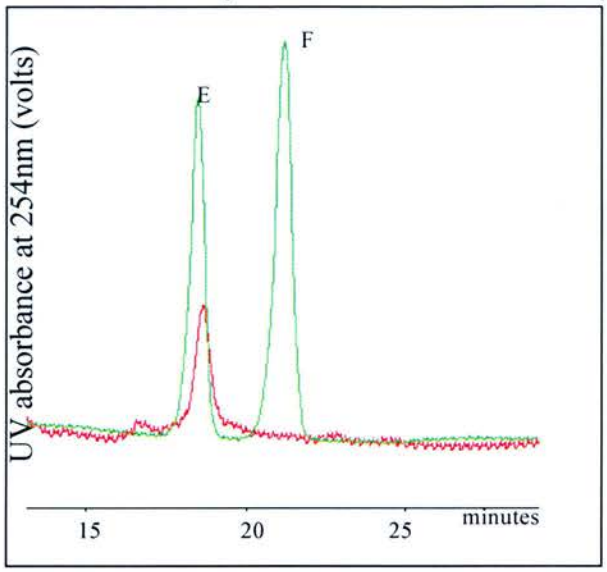


Table 4.2 Absence of 11βHSD reductase activity in 11βHSD 1-/-

Strain	Steroid Impregnated	Cortisol level (ng/g sponge) Ipsilateral steroid Treated sponge	Contralateral placebo Treated sponge
Wild type	Cortisol	4271±186 #	161 ±18
	Cortisone	295 ±25 #**	98 ±19
11βHSD1	Cortisol	3775±1703 #	135 ±46
	Cortisone	87±11	90 ±30

Figure 4.11 and Table 4.2 11βHSD reductase activity in 11βHSD 1-/- mice

Cortisol was not detected in cortisone-impregnated sponges from 11βHSD1 -/- mice. (Figure 4.11) HPLC Chromatogram with uv absorbance detection, green trace represents standard (E cortisone, F cortisol) red trace is sponge extract from cortisone-impregnated sponge from an 11βHSD1 -/- mice. No cortisol was detected in the sample. Table 4.2 Cortisol concentrations in sponge extracts from C57/Bl6 and 11βHSD1 -/- mice. Cortisone was not converted to cortisol in 11βHSD1 -/- mice. Results are mean ± SEM for n=3-6 experiments. # $p<0.01$ versus contra lateral placebo. **$p\leq0.01$ for differences between C57Bl6 and 11βHSD1 -/- .

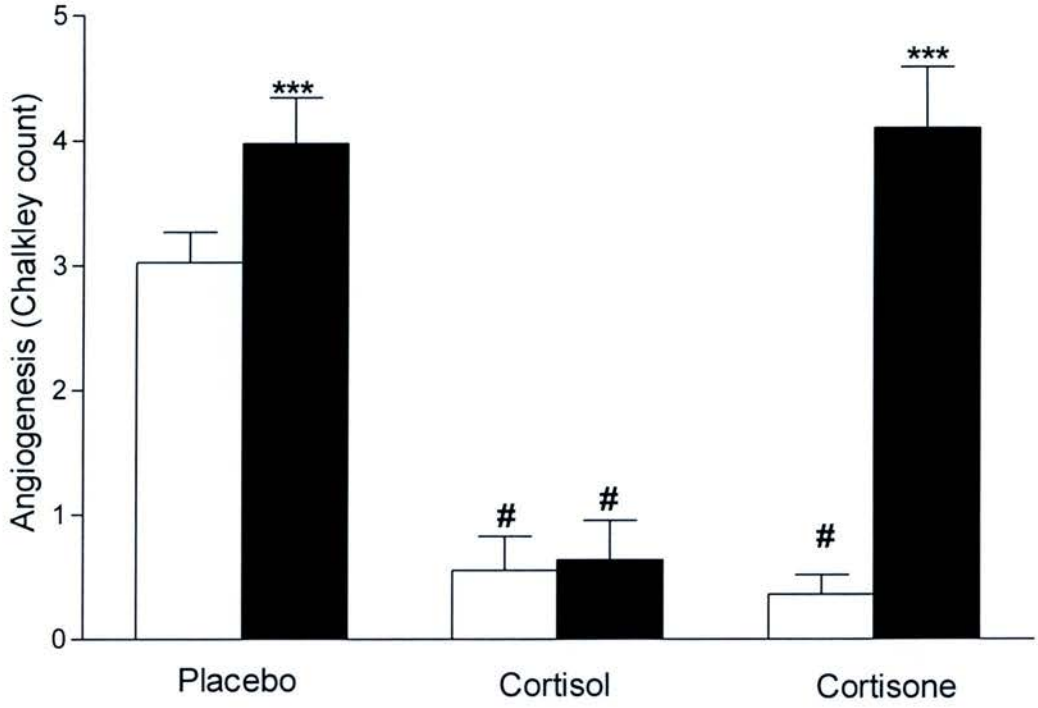


Figure 4.12 11βHSD-1 represses angiogenesis *in vivo*

Sponges from C57Bl6 wild type (open columns n=12) or 11βHSD1 -/- (filled columns n=6) mice with and without glucocorticoids. Results are mean ± SEM. #p<0.001 versus corresponding vehicle. *** p<0.001 for differences between wild type and 11βHSD1 -/-. Placebo impregnated sponges exhibited an increased angiogenic response in 11βHSD1 -/- compared to wild type mice. Angiogenesis was not different between strains in the presence of cortisol but was inhibited by cortisone in sponges from wild type but not 11βHSD1 -/- mice.

4.4 Discussion

The results described in this chapter confirm the findings from chapter 3 that endogenous concentrations of glucocorticoid are sufficient to inhibit angiogenesis in a glucocorticoid receptor-dependent manner. Furthermore, consistent with *in vitro* findings, local glucocorticoid regeneration by 11βHSD1 reductase activity impairs new vessel formation *in vivo*. In addition, in the absence of exogenous steroid, 11βHSD1 inhibited angiogenesis indicating that 11βHSD1 regulates angiogenesis by tonically repressing new vessel formation.

4.4.1 Modification of the sponge implantation angiogenesis assay

Sponge implantation has been performed in rats to demonstrate the effects of therapeutic concentrations of glucocorticoids to influence angiogenesis (Hori *et al.* 1996). However in this model steroids were delivered by a cannula that was sewn into the sponge and fed out through the skin. Drug delivery by this method meant that animals had to be housed singly. Such housing is an acknowledged stressor for mice, causes an elevation of glucocorticoid concentrations and has been associated with delayed wound healing (Glasper & DeVries 2005). Other techniques for drug administration in this assay include daily injection directly into the sponge (Hague *et al.* 2002) or by subcutaneous pellet implantation (Fajardo *et al.* 1992). Subcutaneous injection generates handling and procedure stresses and thus was avoided through the use of subcutaneous pellets. Silicon pellets previously developed to investigate glucocorticoid replacement in adrenalectomised rats (Livingstone *et al.* 2000a) were used to perform pilot angiogenesis studies in the sponge implant model. Steroid release from such pellets is consistent and occurs up to 4 weeks after implantation

(Cleasby *et al.* 2003). Although this type of drug administration helped to localise delivery it was unclear how much drug was present in the environment of the sponges. Measurement of sponge cortisol concentrations indicated that steroid release from the sponge was localised and did not affect contralateral sponges. In the established models (Hori *et al.* 1996; Illanes *et al.* 2002) drug delivery by injection either by cannula or into the sponge was assumed to be localised to the vicinity of the sponge because of the formation of a fibrous capsule around the sponges (Andrade *et al.* 1987a). In those studies a 1-2mm thick, fibrous, collagen-rich capsule consistently grew over the sponge over the first 6 days following implantation; vessel growth within the sponge also proceeded at a predictable rate with a lag phase of 3-4 day after implantation, followed by exponential growth over the next 14-16 days with growth reaching a plateau from day 21 (Andrade *et al.* 1987). Consequently, in the experiments described in this chapter, sponges were excised 3 weeks following implantation.

Similar to the concerns surrounding localisation of drug delivery; validation for the method of using contralateral placebo-impregnated sponges as controls does not appear to have been performed (Hori *et al.* 1996). Thus mice that received no exogenous steroid, but had sponges implanted bilaterally, were compared to mice that received corticosterone on the right flank and placebo on the left. The results from these studies indicated that there was no difference in angiogenesis in the placebo treated sponges. Therefore, placebo and steroid could be assessed in the same mouse. These pilot studies indicated that large concentrations of glucocorticoids inhibited angiogenesis *in vivo* but also that these concentrations had systemic side effects: plasma corticosterone was suppressed, the weights of

glucocorticoid-sensitive organs were decreased and wound healing was impaired. These systemic effects were similar to findings reported from previous work using this concentration of corticosterone (Cleasby *et al.* 2003). The decreased plasma corticosterone is thought to be a consequence of an impaired glucocorticoid stress response secondary to tonic release of steroid from the pellet (Cleasby *et al.* 2003). Concentrations of glucocorticoids were subsequently adjusted to maintain an angiostatic influence but reduce the systemic effects.

Despite the striking changes in angiogenesis seen with high concentrations of corticosterone, the protein assay did not detect any differences between glucocorticoid- and placebo-treated sponges. Protein was quantified as a marker of angiogenesis and the results indicated that this was not a reliable indicator of new vessel formation when measured using the Bradford assay. This particular assay measures the change in light absorption associated with binding of a colorimetric dye (Coomassie Blue G-250) to proteins and has been used extensively because it is highly sensitive and easy to perform (Sapan *et al.* 1999). Protein binding to dye is reliant on protein composition as well as quantity, and factors such as amino-acid content, degree of glycosylation and protein conformation have been shown to alter binding characteristics (Sapan *et al.* 1999). Thus the limitations of the assay rather than the absence of any differences in protein concentrations may explain the data. Other methods of protein quantification may have provided further information but these were not pursued because consistent results were obtained by vessel counting.

Vessels were counted using 2 techniques: either high power field counting or Chalkley count determination. Both methods were simple to perform reliable reproducible and both detected the inhibition of angiogenesis by glucocorticoids.

However Chalkley count is better established (Hague *et al.* 2002; Fox *et al.* 1995) and more time efficient and thus was adopted as the method of choice.

Vessels were identified by haematoxylin and eosin staining as in previous investigations (Andrade *et al.* 1987; Edwards *et al.* 1960). Extensive attempts were made to label endothelial cells within the sponges using either anti-CD31 or anti-vWF antibodies (Datta *et al.* 1995; Newman & Newman 2003). For both of these antibodies rigorous method development of staining protocols, including varying antigen retrieval techniques, altering buffers, incubation times and antibody concentrations failed to establish a satisfactory staining method. More recently a publication examining different endothelial cell markers has indicated that the lack of success may have been associated with the use of 10% formalin fixation, although other groups claim to have success with this (Illanes *et al.* 2002; Ismail *et al.* 2003). Following discussion with a leading angiogenesis investigator vessels were identified using haematoxylin and eosin staining alone although in the light of recent information (Ismail *et al.* 2003) future studies might consider the use of a different fixative agent. Following extensive method development the sponge implant model was used to investigate the influence of endogenous glucocorticoids to regulate angiogenesis.

4.4.2 Endogenous glucocorticoids inhibit angiogenesis

The role of endogenous glucocorticoids was evaluated by performing adrenalectomy immediately prior to sponge implantation. Plasma corticosterone concentrations confirmed the efficacy of surgery and in the absence of measurable plasma corticosterone sponge vascularity increased. Sponges implanted in sham-operated

animals had fewer new vessels in comparison to sponges from adrenalectomised animals. Therefore endogenous glucocorticoids repress angiogenesis and adrenalectomy facilitates sponge vascularisation.

To confirm these findings, but also to determine the receptor responsible for the *in vivo* angiostatic effects of glucocorticoids, sponges were treated with a glucocorticoid or a mineralocorticoid receptor antagonist. The mineralocorticoid receptor antagonist spironolactone had no effect on angiogenesis in the implanted sponges. This in contrast to reported effects suggesting that spironolactone inhibits angiogenesis (Klauber *et al.* 1996; Guggino *et al.* 2003; Guggino *et al.* 2002). However, these reports should be interpreted with caution as the concentrations of spironolactone used were supra-therapeutic. The concentration of spironolactone administered to sponges in the present study was calculated by extrapolating from glucocorticoid doses. The absence of an effect of spironolactone on angiogenesis is in agreement with findings using therapeutic concentrations *in vitro* as observed in aortic ring cultures (Chapter 3).

Glucocorticoid receptor antagonist, RU38486, treatment of impregnated sponges increased angiogenesis, confirming that endogenous glucocorticoids inhibit angiogenesis. In addition, increased sponge vascularity in the presence of a glucocorticoid receptor antagonist indicated that the effects of endogenous glucocorticoids on angiogenesis are glucocorticoid receptor dependent. This is consistent with the findings *in vitro* in aortic ring culture where RU38486 abolished the angiostatic effects of exogenous glucocorticoids.

RU38468 stimulation of angiogenesis in sponges was associated with decreased plasma corticosterone. Clinically RU38486 increases plasma glucocorticoids by inhibiting feedback to the hypothalamic-pituitary–adrenal axis (Byrne *et al.* 2004) . In animal studies RU38486 also increased plasma corticosterone (Wade *et al.* 1988). However, there are reports that RU38486 inhibits adrenal steroidogenic enzymes to impair steroid production (Albertson *et al.* 1994). More recently RU38486 has been associated with impaired responses to recurrent stress, such that plasma corticosterone fails to rise with repeated stress (Moldow *et al.* 2005). Whether inhibition of steroidogenic enzymes or impairment of stress responses accounts for the current results requires further evaluation. It is, however, unlikely that the reduced plasma corticosterone levels are as a consequence of drug cross-reactivity in the radioimmunoassay for this would have led to an increase rather than decrease in the measured concentration of steroid.

Previous studies using RU38486 to explore the effects of endogenous glucocorticoids on wound healing and inflammation have shown conflicting results (Tcacencu 2002; Padgett *et al.* 1998; Bitar *et al.* 1999; Leech *et al.* 2000a; Vinson *et al.* 1998; Tjandra *et al.* 1996; Laue *et al.* 1988; Leech *et al.* 2000b; Kohli *et al.* 1998). RU38486 consistently increases the inflammatory response but the effect of this on wound healing is variable. The different consequences of increasing inflammatory responses may reflect the dissimilarity of *in vivo* models used. Differences in vascularity, for example, may determine the influence of increased inflammatory cell migration and re-vascularisation of inflamed tissue. Although disparate in some of their findings, these studies indicate that endogenous glucocorticoids influence processes associated with angiogenesis with subsequent

effects on tissue healing. Whether this associates with endogenous glucocorticoid regulation of angiogenesis in pathology will require further investigation.

RU38486 is also recognised to be a progesterone receptor antagonist(Yamamoto *et al.* 1994). It is unlikely that progesterone antagonism is responsible for the data presented in this chapter since male mice were used that have low levels of progestogens. In addition, RU38486 antagonism of progesterone receptors would be expected to be associated with an impairment of angiogenesis as progestogens are associated with increased new vessel formation (Hague *et al.* 2002).

4.4.3 11βHSD1 tonically represses angiogenesis

Whether glucocorticoid-mediated regulation of angiogenesis could be influenced by 11βHSD1 was investigated using placebo- or glucocorticoid-impregnated implants in wild type and 11βHSD1 deficient mice. 11βHSD1 reductase activity was confirmed in sponges from wild type mice by using human corticosteroids, cortisol and cortisone, to allow differentiation from endogenous steroids. In the presence of cortisol and cortisone, angiogenesis was inhibited in wild type mice. In contrast in sponges from 11βHSD1 deficient mice reductase activity was absent and, furthermore, although cortisol inhibited angiogenesis the angiostatic effect of cortisone was abolished in these mice. Thus glucocorticoids impair angiogenesis *in vivo* and 11βHSD1 moderates glucocorticoid-induced angiostasis through the regeneration of active glucocorticoids. In addition, in a comparison of placebo-impregnated sponges from wild type and 11βHSD1 deficient mice, 11βHSD1 was found to repress angiogenesis. 11βHSD1 therefore tonically inhibits angiogenesis *in*

vivo and adds a further tier of control to the regulation of angiogenesis by endogenous glucocorticoids.

The tonic regulation of angiogenesis was a novel finding *in vivo* that has not been observed *in vitro* in aortic rings (Chapter 3). This could be a consequence of the systemic factors that are unique to the *in vivo* setting such as the presence of inflammation or the metabolic environment of the implants/subcutaneous tissue or the contribution to angiogenesis that circulating precursor cells may make. Inflammation is closely linked to angiogenesis (Conway *et al.* 2001) and, furthermore, inflammatory cytokines are acknowledged to increase vascular smooth muscle cell 11βHSD1 activity and expression (Cai *et al.* 2001). In addition 11βHSD1 is potentially a key regulator of inflammation resolution not only as a result of local glucocorticoid regeneration but also as regulator of macrophage ability to phagocytose neutrophils. Whether the absence of local glucocorticoid regeneration in the vessel wall or impaired macrophage phagocytic function is sufficient to increase the inflammatory response and alter the angiogenic cascade in 11βHSD1 deficient mice will require further investigation.

Future work to examine the nature of the inflammatory cell infiltrate of sponges may reveal differences between the strains that may help to account for the altered angiogenesis responses. Other groups in the past have analysed sponge fluid for cytokine concentrations to explore whether the observed effects of glucocorticoids might be as a consequence of anti-inflammatory effects (Hori *et al.* 1996). Extracts of sponge homogenates in the studies from this chapter have been stored and could be examined. However, altered levels of VEGF, FGF or cytokines may indicate an effect of steroids or simply the differences in sponge cellularity and further

evaluation of these samples is not currently planned. Application of the sponge technique to other transgenic mice might help to dissect the importance of different components of the angiogenesis cascade. Previously the corneal angiogenesis model has, for example, been applied to severe combined immunodeficient mice or natural killer cell-deficient mice to explore the role of inflammation in angiogenesis (Kenyon *et al.* 1996). Applying the sponge implant model to these mice with steroid impregnated sponges might help to dissect out the anti-inflammatory effects of steroids from their effects on cell migration, proliferation and collagen metabolism. If further studies with sponges were planned it would be beneficial to continue to refine endothelial cell immunohistochemistry techniques. Altering the solution used to fix tissue may help to preserve antigens and therefore also be useful in the attempts to identify inflammatory cells.

In summary, with extensive model development the subcutaneous sponge implant assay has been used to demonstrate that endogenous glucocorticoids inhibit angiogenesis *in vivo.* In addition 11βHSD1, through the regeneration of glucocorticoids, tonically repressed angiogenesis and therefore adds a further tier to the regulation of angiogenesis by glucocorticoids. These data are consistent with *in vitro* findings (Chapter 3) but also indicate a physiological role for glucocorticoids and 11βHSD1 in the regulation of angiogenesis. Sponge implants have been associated with a non-specific immune reaction which could be argued not to a physiological response to injury (Andrade *et al.* 1987). Therefore further work should be performed to examine the influence of glucocorticoids to alter angiogenesis in pathology.

Chapter 5

Prevention of endogenous glucocorticoid reactivation by 11βhydroxysteroid dehydrogenase deficiency influences angiogenesis *in pathology*

5.1 Introduction

Physiologically relevant concentrations of glucocorticoids were found to inhibit angiogenesis and their effects were modulated by vessel wall 11βHSD1 *in vitro* (Chapter 3). Furthermore *in vivo* endogenous glucocorticoids repressed the angiogenic response in sponges, which was also tonically impaired by 11βHSD1 (Chapter 4). However to establish whether these findings are relevant in pathology requires validation in a pathological model of angiogenesis.

Differences exist between *in vivo* models that rely upon infiltration (models such as the subcutaneous sponge, Matrigel or tumour subcutaneous angiogenesis assays) and other methods where a tissue angiogenic response to injury is examined (for example retinal, corneal, cutaneous wound healing or ischaemic limb models). In these latter models tissue insult results in hypoxia or ischaemia (Singer & Clark 1999; Heba *et al.* 2001). Hypoxia may be induced by reducing oxygen tension and ischaemia may be imposed by tissue or arterial injury. In angiogenesis in pathology it is the vascular response of an endogenous tissue to hypoxia or ischaemia and subsequent inflammation that distinguishes these assay from other *in vivo* assays that use foreign body implantation.

Angiogenesis in pathology is part of an organised wound healing response that establishes a new network of blood vessels with a recognised hierarchy (Jain 2003). This type of organised response is in contrast to pathological angiogenesis that is found in primary vascular tumours and other tissue malignancies (Carmeliet & Jain 2000). Vessel formation in the latter situations appears to be dysregulated, vascular organisation is lost, and vessels of different shapes and sizes chaotically assemble

into an inefficient structure. Although the regulation of pathological angiogenesis is important, especially in the development of new chemotherapies, this thesis concerns with the physiological regulation of angiogenesis by glucocorticoids and 11βHSD1. Therefore in this chapter studies are performed examining the effects of tissue ischaemia on angiogenesis in the heart and skin.

Mouse chronic coronary ligation technique is well established (Yang *et al.* 2002; Wang *et al.* 2004; Lutgens *et al.* 1999; Lu *et al.* 2004) and has been used to document the effects of different gene deletions on the myocardial response to ischaemia. The model involves the creation of cutaneous thoracotomy incision that also allows a study to be made of the angiogenic response of the skin from the same animal. Although the mouse model is well established, and despite myocardial revascularisation remaining a therapeutic goal of ischaemic heart disease, little is understood regarding the appearance of new vessels in the ventricle immediately post infarct. An angiogenic reaction has been noted to occur by the presence of increased vessels 7-10 days following infarction and numerous attempts has been made to promote this response to improve revascularisation and ventricular function (Symes *et al.* 1999; Solomon & Gersh 1993; Orlic *et al.* 2001; Michael *et al.* 1999; Hodgson *et al.* 2004; Heymans *et al.* 1999; Helisch & Schaper 2003; Hao *et al.* 2004). In an attempt to ameliorate the inflammatory response in the healing myocardium and salvage myocardiocytes, glucocorticoids have been used therapeutically and in animal studies; however the results from these studies are inconsistent (Kones 1975). It is also not clear whether therapeutic glucocorticoids inhibit angiogenesis in the infarcted tissue.

The data from chapters 3 and 4 indicate that endogenous glucocorticoids might repress angiogenesis in infarcted myocardium and that systemic anti-glucocorticoid therapy may improve myocardial revascularisation. However, the influence of endogenous glucocorticoid to modulate the angiogenic response to myocardial infarction has not been considered previously. An anti-glucocorticoid agents might improve the angiogenic response and potentially improve tissue recovery after infarction, but it would be risky to administer a systemic anti-glucocorticoid at the same time as the significant stressor of myocardial infarction.

However preventing the local regeneration of glucocorticoids by inhibiting 11βHSD1 may provide an alternative strategy targeted to limited tissues. Unfortunately there are no readily available specific inhibitors for 11βHSD1 (Seckl & Walker 2001) but the genetically modified 11βHSD1 deficient mouse provides a useful model to investigate whether local regeneration of glucocorticoids is an important determinant of angiogenesis in pathology.

Therefore the aims of this chapter were to develop 2 assays of angiogenesis in pathology that would enable investigation of the influence of endogenous glucocorticoid to modulate tissue revascularisation. The models were used to examine whether 11βHSD1 was a key determinant of tissue angiogenesis in a pathological setting.

5.2 Methods

Both models underwent extensive method development before applying them to further investigations examining the influence of glucocorticoids on angiogenesis in pathology. Adaptations were made to surgical technique, length of experiments, vessel identification and assessment of revascularisation and are all detailed below.

5.2.1 Chronic coronary artery ligation

(Refer to section 2.9) Myocardial infarction in rodents can be induced in recovery surgical procedures through injuring a major coronary artery by several different mechanism including suturing (Li *et al.* 1997), cauterization (Moskowitz *et al.* 1979), or cryo-injury (Ciulla *et al.* 2004; Huwer *et al.* 1999). More recently ischaemia reperfusion models in mice have been developed with the aim of better reflecting the clinical experience of infarct and revascularisation (Michael *et al.* 1999). However chronic coronary ligation was selected for these studies because of the reliability of this technique to reproduce large infarcts in a predictable territory and generate a sustained hypoxic insult that would stimulate myocardial angiogenesis (Lutgens *et al.* 1999).

Mice- Male, C57Bl6J wild type controls and 11βHSD-1 homozygous null (-/-) mice aged 8-10 weeks were used (Charles River, UK)

Surgery- Expertise in the surgical techniques required for these models was obtained under the supervision of Dr Isam Sharif (Centre for Cardiovascular Science Edinburgh University) and practiced using non-recovery procedures on anaesthetised

animals. A formal assessment of the technique was made by Dr Jane Conole, University of Edinburgh Veterinary Surgeon.

Mice were anaesthetised by intraperitoneal injection of xylazine (0.018mg/kg), ketamine (100mg/kg), and atropine (600mcg/kg) (Mora *et al.* 2003). Surgery was performed as described in the methods (Section 2.9.2) (Lutgens *et al.* 1999). Briefly following endotracheal intubation and mechanical ventilation, superficial tissues were dissected, a 2cm incision made in the 4^{th} intercostal space, the pericardium divided and the left main descending artery ligated with 6.0 prolene suture. In sham operated animals the suture was not ligated. The thoracic wall was closed by layered suturing; the skin was stitched with a continuous suture using 5-0 Mersilk with a 10mm 3/8c round-bodied needle. On completion of surgery animals received intraperitoneal atipamazole (5mg/kg) and subcutaneous buprenorphine (0.05mg/kg). At the end of the experiment animals were re-anaesthetised, underwent echocardiography followed by intracardiac catheterisation for pressure measurements and intra-cardiac blood collection and were sacrificed. Excised hearts and cutaneous wounds were processed for histology.

Experiment length- Initially, to record the timing of new vessel appearance in infarcted myocardium, animals were sacrificed on days 1, 3, 5, 7 or 14. These initial data indicated that angiogenesis was well established by day 7 and this time point was adopted as the standard experiment length.

Histology- At excision hearts were bisected, weighed and placed in 10% formalin and processed for histology by paraffin embedding and sectioning at 8μm (Mora *et al.* 2003)(See section 2.8.5). Sections were subsequently stained with haematoxylin and eosin to measure infarct size, van Gieson stain to record the deposition of collagen and immunohistochemistry techniques to identify endothelial cells.

Endothelial cell identification- Rat anti-mouse anti-CD 31 antibodies (Pharmingen) were initially used to identify endothelial cells. However labeling with this antibody was not satisfactory despite repeated attempts to refine the methods by altering antigen retrieval techniques, antibody concentrations, incubation times and buffers (see Section 2.9.5). Instead, rabbit anti-human von Willebrand factor antibodies (Dakocytomation, UK) were used and consistent staining was observed using primary antibody dilutions of 1:200. Staining for von Willebrand factor detected small and medium size vessels but did not detect the microvasculature. Nevertheless it was possible to document an angiogenic response to infarction with time using these antibodies and therefore this technique was used as the standard protocol for these experiments.

Quantification of angiogenesis - Vessels were counted at x 400 magnification in the 4 most vascular fields, (2 endocardial, 2 epicardial), using a 0.0625mm^2 reticule; the borders of the reticule were within the infarct as previously described (Virag & Murry 2003). The numbers of small-medium size vessels counted using this technique were similar to previous observations (Hao *et al.* 2004).

Infarct size measurement (see section 2.9.8) Infarct size was determined as a percentage of left ventricular area. It was quantified by measuring the area affected by infarction as indicated by the region of disrupted myocardial architecture, proliferating cells and inflammatory cell infiltrate on haematoxylin and eosin stained sections (Virag & Murry 2003). Measurement was performed using captured images (Research Systems Photometric camera) from light microscopy and analysed using in house scripts (Lutgens *et al.* 1999).

Assessment of left ventricular remodelling-Mouse echocardiography was performed to examine the effect of infarction and treatment on left ventricular parameters (See Section 2.9.3). Before sacrifice animals were re-anaesthetised and echocardiography was performed using a Diasus ultrasound machine (Dynamic Imaging Livingston UK) (Mora *et al.* 2003). Images were stored on an optical disk and analysed offline using Diasus software (Dynamic Imaging). Left ventricular (LV) parameters (see Table 5.1) were measured at the mid- papillary level on live 2-D images and averaged from 2 cardiac cycles. Left ventricular function as assessed by ejection fraction (EF) ([(LVEDA-LVESA)/LVEDA] x 100) and fractional shortening (FS) ([(LVEDD-LVESD)/LVED] x 100) (Mora *et al.* 2003) was quantified as a measure of myocardial performance.

Left Ventricular Parameters	Abbreviation
left ventricular end diastolic diameter	LVEDD
left ventricular end systolic diameter	LVESD
left ventricular end diastolic area	LVEDA
left ventricular end systolic area	LVESA
posterior wall thickness at end diastole	PWD
posterior wall thickness at end systole	PWS
ejection fraction	EF
fractional shortening	FS

Table 5.1 Left ventricular parameters measured by echocardiography

Left ventricular (LV) parameters were measured at the mid- papillary level on live 2-D images and averaged from 2 cardiac cycles. Images were stored on an optical disk and analysed offline using Diasus software.

Left Ventricular Pressures Dr Isam Sharif performed these procedures. In preliminary studies following echocardiography, the right carotid artery was cannulated using a pressure-transducer tipped catheter (1.4F, Millar Instrument Inc, USA) for measurement of blood pressure and heart rate. The catheter was then advanced into the left ventricle for measurement of left ventricular pressure. The left ventricular diastolic pressure was not raised in mice that had undergone chronic coronary ligation procedures 7 days previously as previously noted (Lutgens *et al.* 1999) therefore this technique was not used in further experiments.

5.2.2 Cutaneous Wound Healing Assay

Thoracotomy wounds from mice undergoing chronic coronary ligation that had been sutured with 5.0 mersilk (Ethicon, Johnson & Johnson, UK) using a 1mm round-bodied needle were used in these studies. At sacrifice 7 days following chronic coronary ligation the thoracotomy cutaneous wounds were removed by wide excision, placed in 10% formalin and processed for histology. Paraffin embedded skin was sectioned (8μm) and stained with haematoxylin and eosin for morphological analysis or with an endothelial cell marker to quantify angiogenesis. As in the infarcted myocardium anti-CD31 (BD Biosciences, UK) did not satisfactorily identify endothelial cells despite considerable method development. Small and medium sized vessels were identified successfully using anti-von Willebrand antibodies (Dakocytomation, UK).

Quantification of Angiogenesis in cutaneous wounds Thoractomy wounds from mice (in both placebo versus RU38486 treatment groups, or wild type versus 11βHSD-1 -

/- mice groups) were used. Vessel density was determined in the dermis at x250 light microscopy using the mean of triplicate Chalkley counts on 2 sections per wound (Fox *et al.* 1995).

5.2.3 Influence of endogenous glucocorticoids and 11βHSD1 on angiogenesis in pathology

RU38486 (a glucocorticoid receptor antagonist) was used to investigate the endogenous effects of glucocorticoids on myocardial revascularisation and wound healing angiogenesis. Silastic pellets impregnated with 5.25mg of RU38486 or placebo were prepared as described in Chapter 2. The pellets were implanted through a 0.5cm incision on the back of the mouse under halothane anesthesia. The incision wound was sutured closed with 5.0 mersilk (Ethicon, Johnson & Johnson, UK). A week later the mice underwent chronic coronary artery ligation. Animals were sacrificed 7 days post coronary artery ligation. Prior to sacrifice animal were anaesthetised, underwent echocardiography and had blood collected by intra-cardiac puncture for the measurement of plasma corticosterone and RU38486 concentrations. Cutaneous thoracotomy wounds and hearts were excised and processed for histology.

Plasma Analysis- (See section 2.8.7) Blood was collected into a heparin coated syringe, and transferred to a 1.5ml eppendorf on ice, centrifuged and the plasma removed and stored at minus 80^{O}C. Plasma corticosterone was quantified by radio-immuno-assay (section 2.8.7) (Cleasby *et al.* 2003).

Plasma RU38486 was assayed with liquid chromatography with mass spectroscopy (LCMSMS) (section 2.9.6) detection monitoring for an ion characteristic of

RU38486. For this assay, the range of detection was <30ng/ml to >0.5ng/ml. Dr Natalie Homer performed the LCMSMS quantification technique.

11βHSD1 regulation of angiogenesis in pathology- Coronary artery ligation or sham procedures were performed on male aged matched C57Bl6J and 11βHSD-1 homozygous null (-/-) mice. Mice were sacrificed 7 days following surgery. Prior to sacrifice mice were re-anaesthetised and underwent echocardiography. At sacrifice hearts and cutaneous thoracotomy wounds were excised and processed. Skin was placed in 10% formalin. Hearts were bisected by cutting through the long axis of the heart through middle of the infarct; half was stored in 10% formalin for histology, half was placed immediately on to dry ice and stored at $-80^{O}C$ for an 11βHSD activity assay.

11βHSD activities in mouse heart- (See section 2.9.7)

An assay developed for use in tissue homogenates was used to assess 11βHSD activity in the heart (Jamieson *et al.* 1995). Both reductase and dehydrogenase activities were assessed (Sandeep & Walker 2001; Cai *et al.* 2001). Heart tissue from C57Bl6 mice that had undergone coronary artery ligation or sham surgery 7 days previously was placed in buffer and homogenized. 10µl of homogenate was taken for protein quantification by the Bradford assay (Section 2.8.6). Sample protein concentration was used to standardize the amount of sample added to the assay. Tritiated steroid was added to the appropriate co-factor and a buffered solution of sample such that the final concentration of sample was 0.2mg protein/ml. ^{3}H-

11dehydrocorticosterone and cofactor NADPH were used for the reductase reaction; the dehydrogenase response was driven by ^{3}H-Corticosterone and cofactor NAD. Samples were incubated in a water bath at 37^{0}C for 2.5 or 5 hours for the reductase reaction and 5 or 16 hours for the dehydrogenase response. Steroids were extracted with ethyl acetate, dried, resuspended in ethanol, separated by TLC and estimated by scintillation counting. Enzyme activity in each direction was expressed as the percent conversion to product (Jamieson *et al.* 1995).

5.2.4 Statistics

Data are mean ± SEM. Comparisons were made by ANOVA with least squares difference post hoc tests. Inter-assay- and intra-assay coefficients of variation in wild type mice were 19 % (n=11) and 10 % (n=11) in day 7 infarcts and 7% (n=4) and 12% (n=4) for day 7 wounds. Intra-assay variation was measured by calculating the coefficient of variation between readings for each section and finding the mean of this value. Inter-assay variation was calculated by dividing the standard error of the means by the standard deviation of the means and expressed as a percentage.

5.3 Results

5.3.1 Method development: establishing standard procedures

A thorough programme of education and practice of anesthetics and surgical techniques supplemented module 4 of the Personal Licensee Training Course and was completed over 20 weeks. At the end of this period the pilot study to determine the appearance of new vessels with time was performed. From this study data was also obtained regarding operative morbidity and mortality.

Operative morbidity and mortality- Average weight loss in C57Bl6 mice was greatest 3 days following coronary artery ligation (Figure 5.1). Pre-operative weight was regained at day 5 in sham operated animals and between days 8-14 in animals under going coronary artery ligation. There were no operative deaths in the sham group in the coronary artery ligated animals mortality was 18% (7/39).

Endothelial cell Identification- Anti von Willebrand factor antibodies labelled endothelial cells lining blood vessels in the myocardium and skin (Figure 5.2a-c) and was used to document the appearance of new vessels.

Experiment Length- The myocardial vascularity of sham operated animals was consistent at each of the time points examined and appeared not to be influenced by the surgery (Figure 5.3). In coronary artery ligated hearts vascularity increased with time until day 7. This time point was used to measure angiogenesis in the remaining studies. Collagenisation of infarcted tissue was also documented in a qualitative pilot study using Van Gieson staining. Collagen deposition was not observed until day 5 (Figure 5.4).

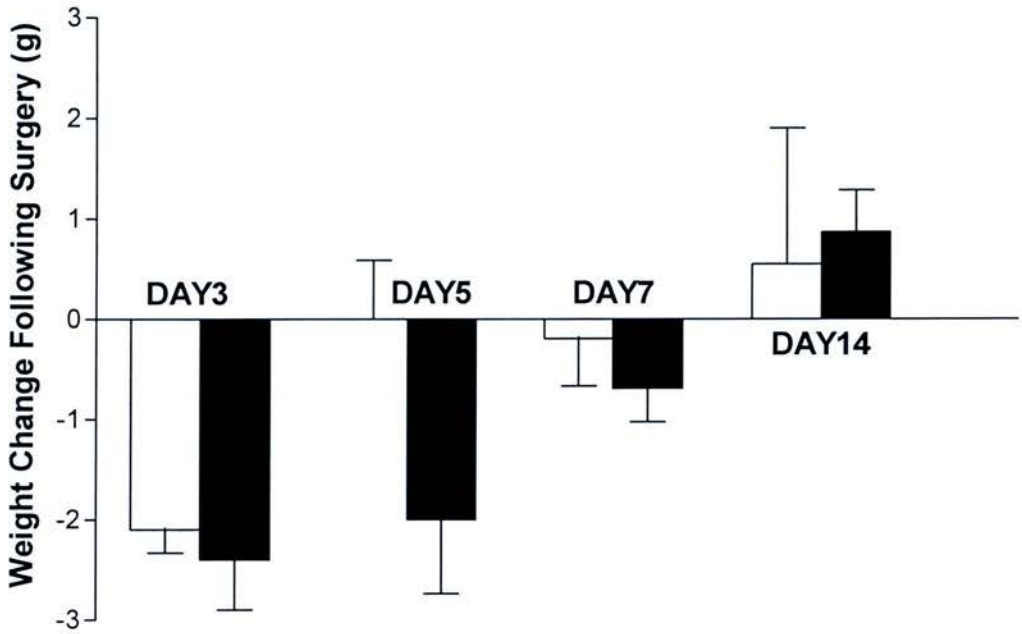
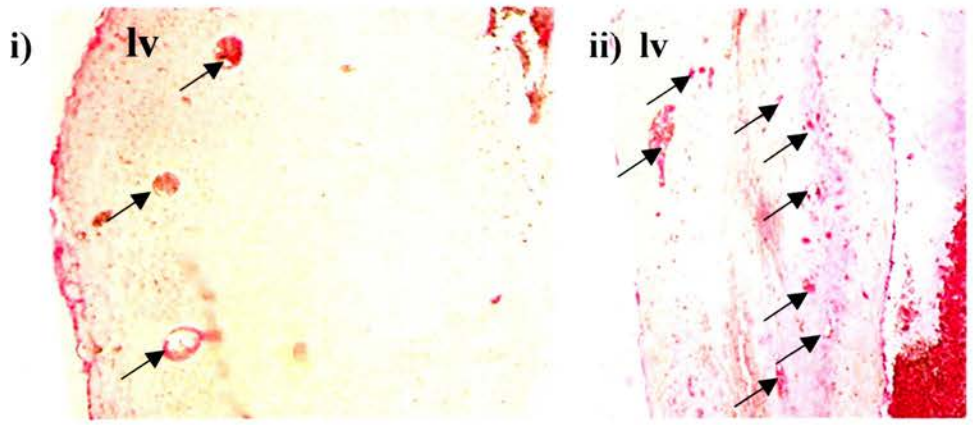


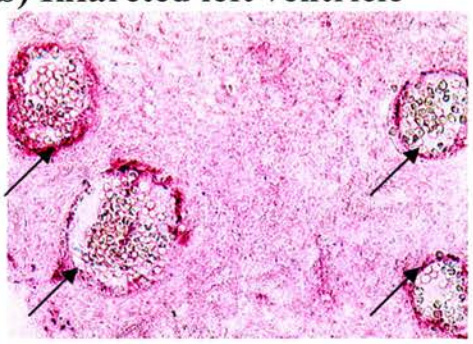
Figure 5.1 Coronary artery ligation or sham operation and body weight

(A) Body weight was reduced following coronary artery ligation (filled bars) or sham (open bars) procedure in C57Bl6 mice (n=18) . Reduction was marked in both groups at day 3 but animals returned to pre-operative weight earlier following sham procedure. Weight gain in excess of pre-operative weight occurs between days 8-14. Data are expressed as mean ± standard error.

a)Sham (i) and infarcted (ii) left ventricle



b) Infarcted left ventricle



c) Thoracotomy wound skin

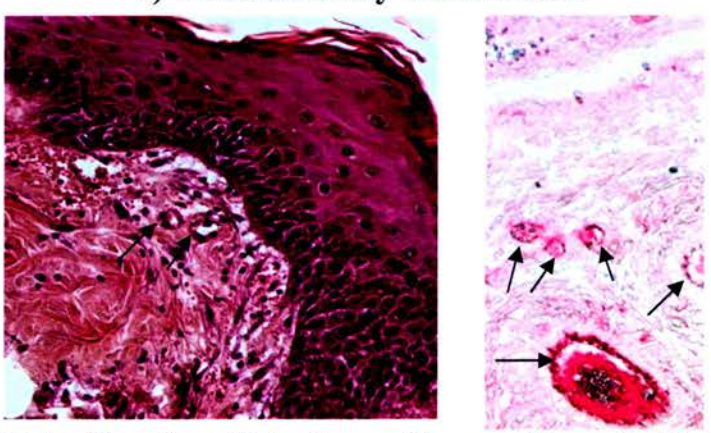


Figure 5.2(a-c) Identification of blood vessel in paraffin embedded sections

Anti-von Willebrand factor antibodies with fast red chromogen labelling of endothelial cells in heart (a-b) and skin (c) 7 days following surgery. (a) (x50) sham (i) and infarcted (ii) left ventricle (lv). Scattered large vessels (black arrows) are seen in thick wall of sham left ventricle(i), in contrast to thin infarcted wall of left ventricle (ii) numerous small vessel are seen (black arrows). (b) High power x100 view of infarcted left ventricle numerous vessel are seen (black arrows). (c) Skin from thoracotomy wounds (i) haematoxylin and eosin cross section (x50) (ii) endothelial cells within vessels (black arrows) (x100).

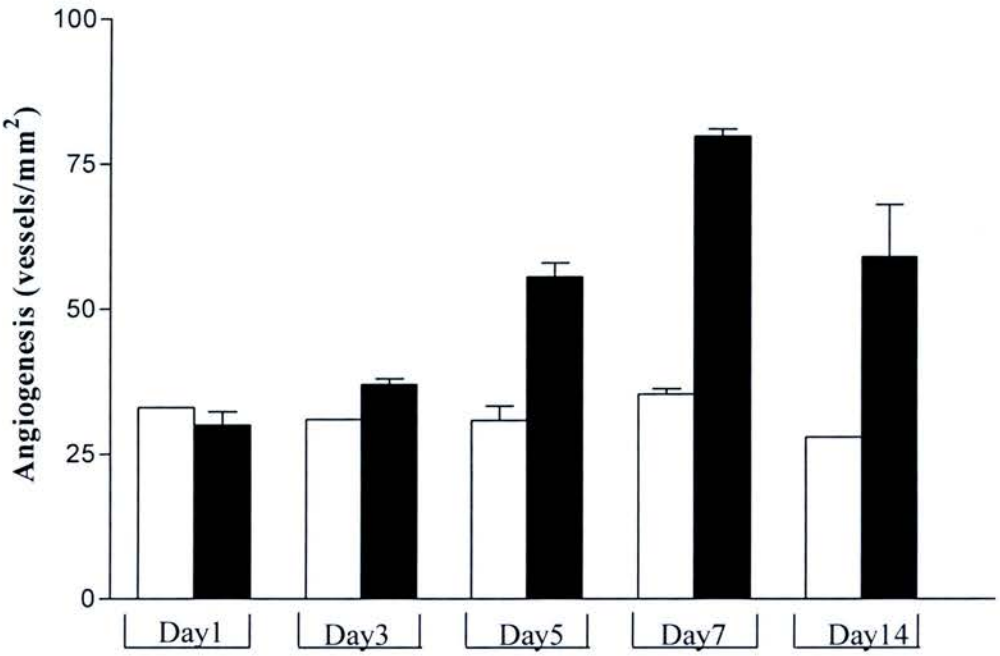


Figure 5.3 Angiogenic response to coronary artery ligation

Vascularity of myocardium following coronary artery ligation (filled columns n=3-11) or sham surgery (open columns n=2-6) in C57Bl6J mice. Sham operated animals showed a constant vascularity in contrast to ligated animals in which vessel counts were increased such that angiogenesis was well established by day 7. Data are expressed as mean ± SEM.

a) Left ventricle day 3 post infarction

b) Left ventricle day 5 post infarction

c) Left ventricle day 7 post infarction

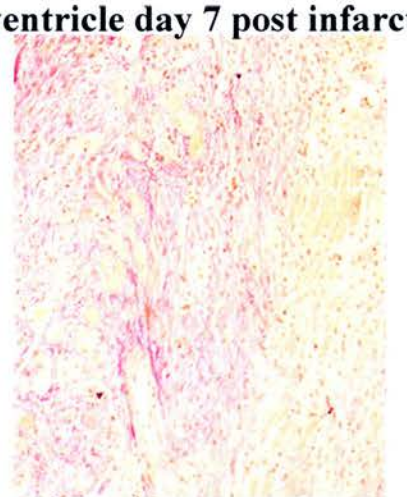


Figure 5.4 Van Gieson staining of infarcted left ventricle

Left ventricular sections from day 3 (a), day 5 (b) and day 7 (c) following coronary artery ligation in C57Bl6 mice. Van Gieson stain colours collagen pink. A dramatic increase in collagen deposition is seen by day 7.

5.3.2 Application of methods: Endogenous glucocorticoids inhibit angiogenesis in pathology

Morbidity and Mortality- RU38486 or placebo pellet implantation was tolerated well; there was no difference in weight change between the 2 treatment groups prior to coronary artery ligation (0.67±0.54g for RU38486 treated animals n=9 versus 0.22±0.25g for placebo treated animals n=4 p=0.16). Both treatment groups underwent chronic coronary ligation surgery no sham procedures were performed. More mice in the RU38486 treated group died following coronary artery ligation than in the placebo treated group, however the study was not powered to detect a significant difference in mortality with treatment (5/9 deaths treated with RU38486, 4 of which were from ventricular rupture: versus 1/4 treated with placebo as a result of ventricular rupture; p= 0.34). Weight change following surgery was similar in both treatment groups (-1.9±1.6g for placebo (n=3) compared to -0.65±1.8g for RU38486 (n=6) p=0.44).

Plasma Analysis- Corticosterone concentration in plasma was higher in placebo treated animals that had undergone coronary artery ligation (487±24nM for placebo treated animals versus 253±27nM for RU38486 treated animals n=4 $p<0.01$) compared to RU38486 treated animals.

In plasma samples processed for quantification of RU38486, alfaxalone was successfully recovered from control and treatment group plasma samples. RU38486 was only detected in samples from animals that had received RU38486 treatment in which the concentration was 12.6±2.68ng/ml (n=4).

Infarct size- At day 7 the proportional area of the left ventricular myocardium affected by coronary artery ligation was similar in both treatment groups (41.8±6.2% for vehicle, 45.5±4.8% for RU38486, n=3-6).

Left Ventricular Dimensions and Function - Compared to vehicle, RU38486 treatment did not alter the influence of coronary artery ligation on LV parameters (Table 5.2). Left ventricular ejection fraction (EF) and fractional shortening (FS) were similar in RU38486 and vehicle treated controls (Table 5.2).

Ventricular Angiogenesis Myocardial revascularisation was enhanced in RU38486 treated mice in comparison to vehicle treated controls (Figure 5.5).

Cutaneous Wound Healing –Thoractomy wounds from placebo and RU38486 treated mice exhibited no differences in gross appearance. Hair re-growth appeared to be similar as was the extent of granulation tissue; wound dehiscence did not occur.

Cutaneous Angiogenesis New vessel formation in the dermis of cutaneous wounds from RU38486 treated mice was enhanced in comparison to vehicle treated controls (Figure 5.6).

Treatment	Vehicle	RU38486
Surgery	INFARCT (n=3)	INFARCT (n=4)
LVEDD (mm)	4.54±0.2	5.1±0.2
LVESD (mm)	3.5±0.2	4.2±0.3
LVareaED	23.3±1.7	27.5±3.2
LVareaES	17.7±1.9	21.4±2.9
PWD (mm)	0.7±0.1	0.4±0.1
PWS (mm)	0.8±0.1	0.7±0.1
FS	22.7±4.9	19.3±3.2
EF%	24.3±3.8	22.6±1.6

Table 5.2 Left ventricular dimensions following coronary artery ligation in vehicle and RU38486 treated mice

There was no difference in left ventricular (LV) parameter between vehicle and RU38486 treated mice. Left ventricular ejection fraction and fractional shortening were similar in the 2 treatment groups. (Results are mean ± SEM, n=3-4). Left ventricular end diastolic diameter (LVEDD), LV end systolic diameter (LVESD), LV end diastolic area (LVEDA), LV end systolic area (LVESA), posterior wall thickness at end diastole (PWD) posterior wall thickness at end systole (PWS)]. Left ventricular ejection fraction (EF) ([(LVEDA-LVESA)/LVEDA] x 100) and fractional shortening (FS) ([(LVEDD-LVESD)/LVED] x 100).

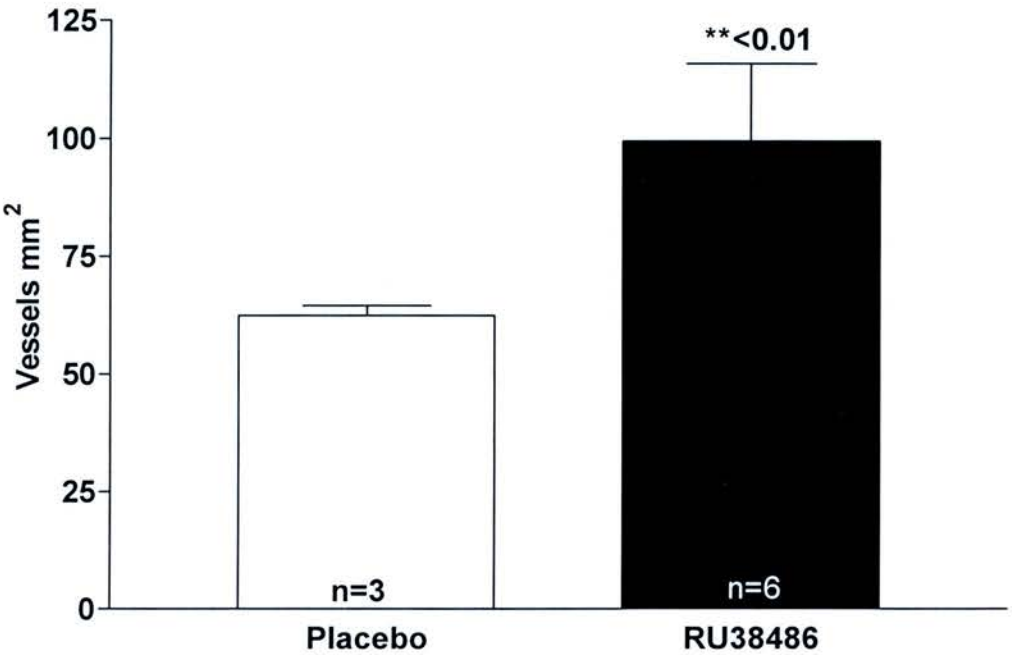


Figure 5.5 Ventricular angiogenesis following myocardial infarction

C57Bl6 mice treated with RU38486 (solid bar) had greater new vessel formation following chronic coronary ligation than placebo treated controls (clear bar). Data are mean±SEM $p<0.01$ by ANOVA, n=3-6.

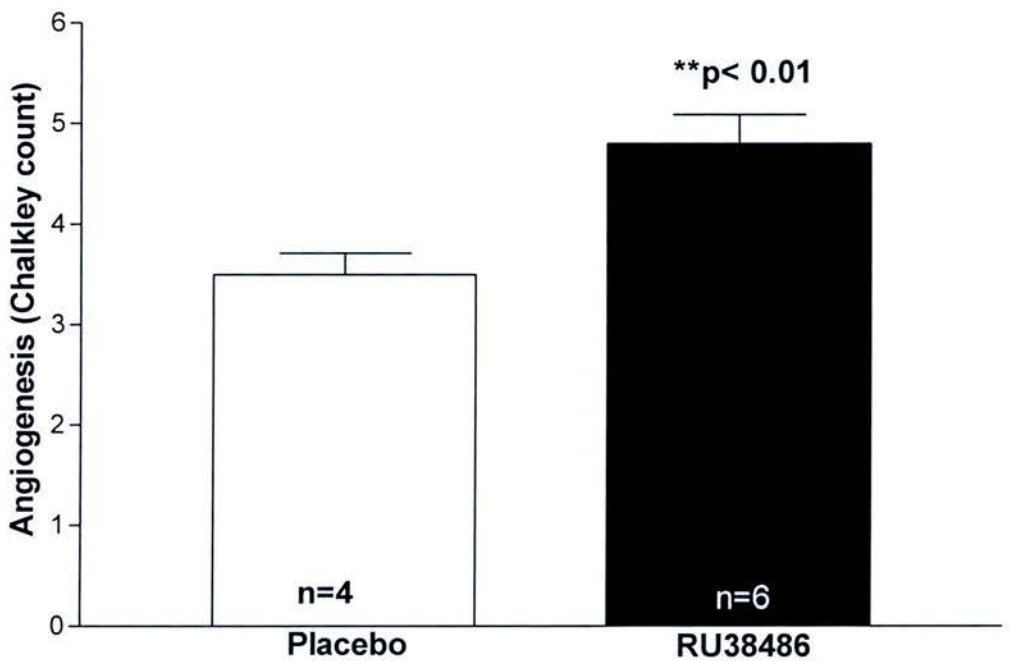


Figure 5.6 Angiogenesis in cutaneous wounds

Thoracotomy skin wounds from animals that had undergone coronary artery ligation 7 days previously. No visible differences were seen in the amount of tissue granulation, hair re-growth or wound dehiscence. However dermal angiogenesis was increased in mice treated with RU38486 (solid bar) in comparison to placebo (clear bar). Data are mean±SEM n=4-6 $p<0.01$.

5.3.3 Application of methods: 11βHSD1 inhibits angiogenesis in pathology

Morbidity and Mortality- 11βHSD1 deficient mice have not been found to have any abnormalities of vascular development, vascular function or congenital heart disease compared to wild type mice (Hadoke *et al.* 2001; Kotelevtsev *et al.* 1997).

Weight reduction at day 7 post operatively in sham and coronary artery ligated animals was greater in 11βHSD-1 -/- mice in comparison to wild types (Figure 5.7). However, there was no difference in mortality at day 7 between strains following coronary artery ligation (18% (7/39- 3 of which were due to ventricular rupture) in C57Bl6 versus 18.75% (3/16- 2 of which were due to ventricular rupture) in 11βHSD1 deficient mice); there was 1 death in sham-operated animals, which occurred in an 11βHSD1 deficient mouse.

11βHSD activity- Reductase and dehydrogenase activity was detected in homogenates of hearts from C57Bl6 mice that had undergone chronic coronary ligation (n=6) and sham procedures (n=4). Activity increased with time for both reaction directions ($p<0.03$ for reductase assay n=10, $p<0.01$ for dehydrogenase assay n=10 analysed by repeated measures ANOVA). Infarction did not influence 11βHSD activities in comparison to sham procedure (Figure 5.8).

Infarct size- At day 7 the proportional area of the left ventricular myocardium affected by coronary artery ligation was similar in both strains of mice (44.2±3.4% for C57Bl6 and 44.2 ±2.6% in 11βHSD1 deficient mice n=5).

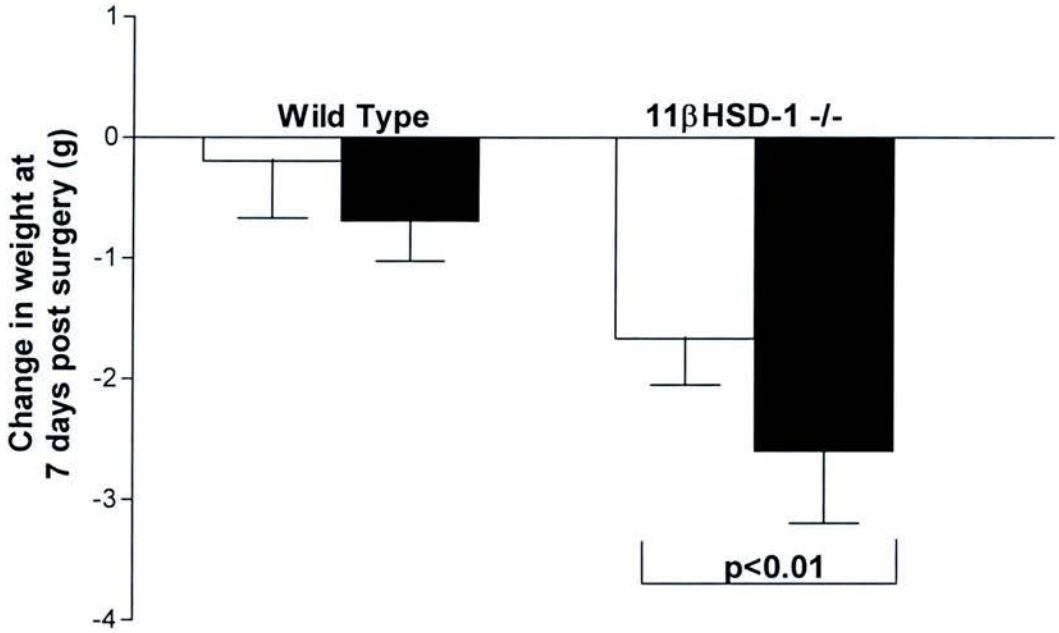


Figure 5.7 Influence of 11βHSD1 on weight loss following surgery

Weight loss recorded at 7days post procedure. Both stains of mice undergoing sham (clear bar) or chronic coronary artery procedure (solid bar) lost weight. 11βHSD-1deficient (11βHSD1 -/-, n=15) had a greater reduction in weight compared to C57Bl6 (wild type n=18) mice. Data are expressed as mean ± SEM; $p<0.01$ by ANOVA for the difference between strains.

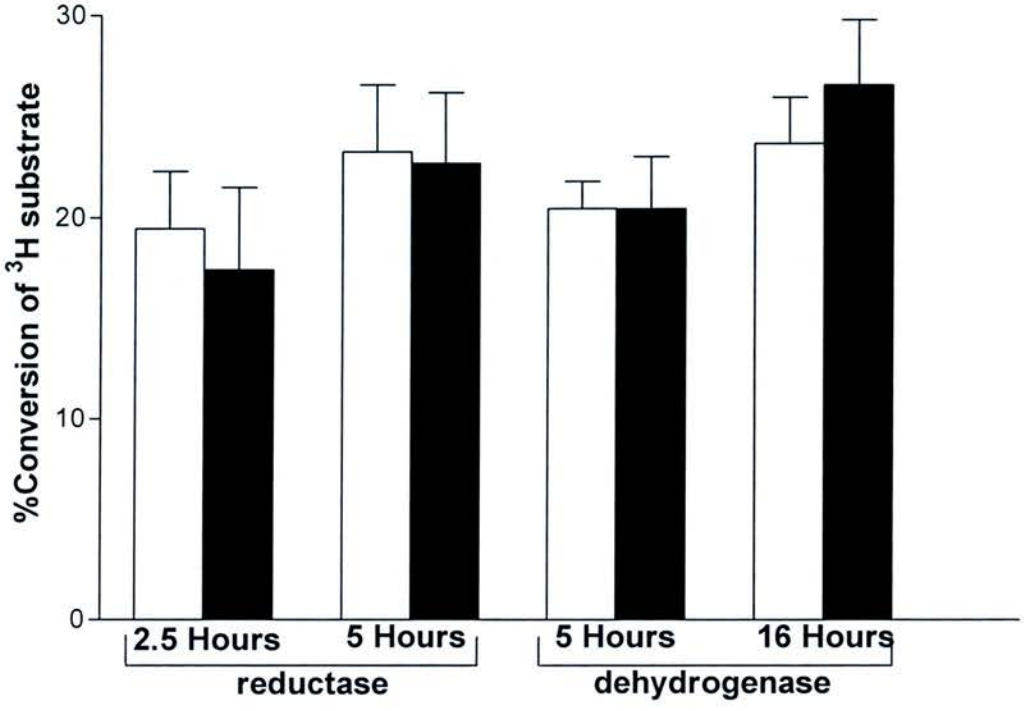


Figure 5.8 Effect of Coronary Artery Ligation on 11βHSD Activity

11βHSD activities are expressed as the percentage conversion of [^{3}H]-substrate by homogenates of mouse heart at standard protein concentrations (n =10). 11βHSD reductase activity was measured after 2.5 and 5 hours incubation; 11βHSD dehydrogenase activity was measured after 5 and 16 hours incubation.

The conversion of ^{3}H substrate increased with time in both reductase ($p<0.03$ by repeated measures ANOVA; n=10) and dehydrogenase ($p<0.01$ by repeated measures ANOVA; n=10) reactions. Surgical treatment with coronary artery ligation (filled columns n=6) in comparison to sham (open columns n=4) did not alter reductase or dehydrogenase 11βHSD reactions.

Left Ventricular Dimensions and Function - There was no difference in baseline left ventricular (LV) parameters between sham-operated C57Bl6 and 11βHSD1 deficient mice. In comparison to sham operation, coronary artery ligation affected all measured parameters of LV dimensions in C57Bl6 and 11βHSD1 deficient mice (Table 5.3). Coronary artery ligation was associated with left ventricular dilatation and thinning of the left ventricular posterior wall in both strains. Coronary artery ligated 11βHSD-1 deficient mice had reduced thinning of the posterior wall and less dilation of the LV cavity in comparison to wild type controls (Table 5.3). Left ventricular ejection fraction (EF) and fractional shortening (FS) were reduced by coronary artery ligation in wild type and 11βHSD1 deficient mice but the reduction was less in 11βHSD-1 deficient mice (Figure 5.9).

Ventricular Angiogenesis - No difference was recorded in myocardial vascularity between sham-operated C57Bl6 and 11βHSD1 deficient mice. In contrast following coronary artery ligation 11βHSD1 deficient mice exhibited enhanced revascularisation in comparison to controls (Figure 5.10).

Cutaneous Wound Healing- Thoracotomy wounds from C57Bl6 and 11βHSD1 deficient mice 7 days following the procedure on gross inspection appeared similar. No differences were noticed in coat re-growth, extent of granulation tissue or wound dehiscence.

Angiogenesis The dermal angiogenic response was greater in 11βHSD1 deficient mice (5.1±0.27 Chalkley count) in comparison to C57Bl6 controls (3.5±0.25 Chalkley count; n=6 $p<0.01$. by ANOVA).

Strain	Wild Type		11βHSD1	
Treatment	Sham (n=7)	Infarct (n=10)	Sham (n=6)	Infarct (n=9)
LVEDD (mm)	3.4±0.1#	4.8±0.1	3.5±0.3#	4.3±0.2*
LVESD (mm)	2.1±0.1#	3.8±0.1	2.1±0.2#	3.2±0.2**
LVareaED	15.9±1.1#	24.4±1.3	13.6±1.0#	17.9±0.9**
LVareaES	6.6±0.7#	17.7±1.4	4.5±0.5#	11.1±0.8**
PWD(mm)	1.0±0.1#	0.7±0.1	1.3±0.1#	1.1±0.0**
PWS(mm)	1.4±0.1#	0.9±0.1	1.7±0.0 #	1.4±0.0**
FS	37.0±2.8#	18.7±2.1	38.5±2.5#	24.7±2.4**
EF%	58.0±3.5#	27.8±3.2	66.3±3.7#	38.8±3.2*

Left ventricular end diastolic diameter (LVEDD), LV end systolic diameter (LVESD), LV end diastolic area (LVEDA), LV end systolic area (LVESA), posterior wall thickness at end diastole (PWD) posterior wall thickness at end systole (PWS)]. Left ventricular ejection fraction (EF) ([(LVEDA-LVESA)/LVEDA] x 100) and fractional shortening (FS) ([(LVEDD-LVESD)/LVED] x 100)

Table 5.3 Left ventricular dimensions in wild type and 11βHSD-1 -/- mice

There was no difference in baseline left ventricular (LV) parameters between sham-operated wild type and 11βHSD-1 -/- mice. Coronary artery ligation influenced all measured indices of left ventricular dimensions and decreased left ventricular ejection fraction and fractional shortening in comparison to sham procedure in wild type and 11 βHSD-1 -/- mice. Relative to wild type controls 11βHSD-1 -/- mice that received coronary artery ligation had relative preservation of many left ventricular parameters including left ventricular ejection fraction. (Results are mean ± SEM n=6-10. # $p<0.01$ versus corresponding infarct. **$p\leq0.01$ or *$p\leq0.05$ for differences between wild type and 11βHSD1 -/-).

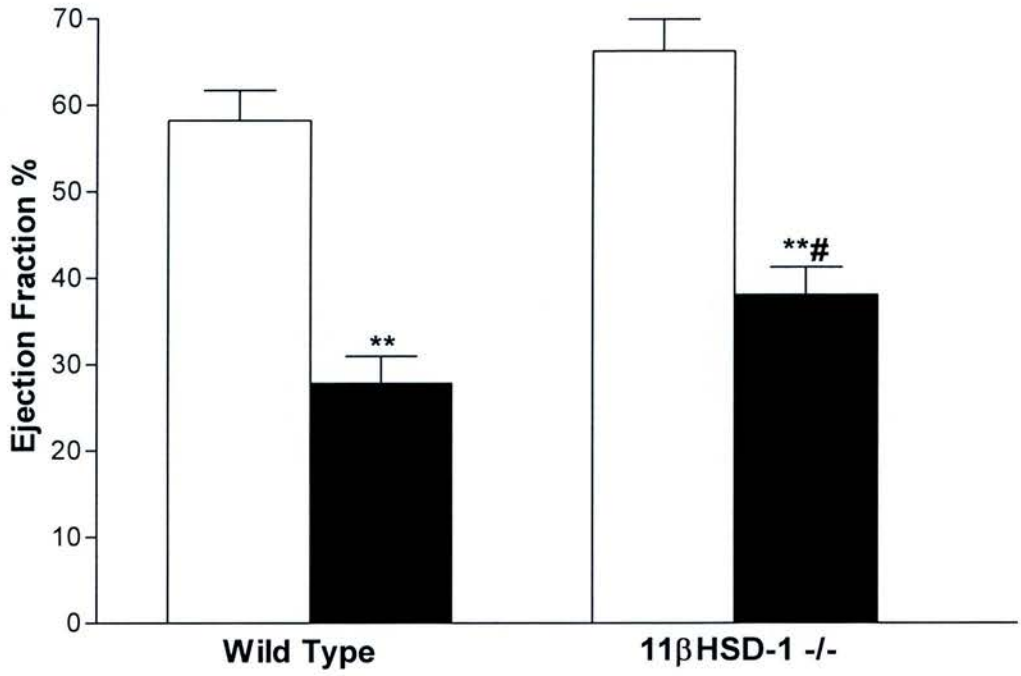


Figure 5.9 Left ventricular ejection fraction (EF)

Left ventricular function was quantified as ejection fraction (EF) by the equation [(LVEDA-LVESA)/LVEDA] x 100 where LVEDA and LVESA represent left ventricular end diastolic and end systolic areas respectively. Coronary artery ligation (filled columns) in C57Bl6 (wild type) and 11βHSD1 deficient (11βHSD1-/-) mice decreased EF in comparison to sham (open columns) (wild type n=6 sham, n=10 ligations and 11βHSD1 -/- n=5 sham, n=9 ligations). Relative to wild type controls 11βHSD-1 -/- mice that received coronary artery ligation had relative preservation of left ventricular EF. (Results are mean ± SEM. ** $p<0.01$ versus corresponding sham; # $p\leq0.01$ for differences between wild type and 11βHSD1 -/-).

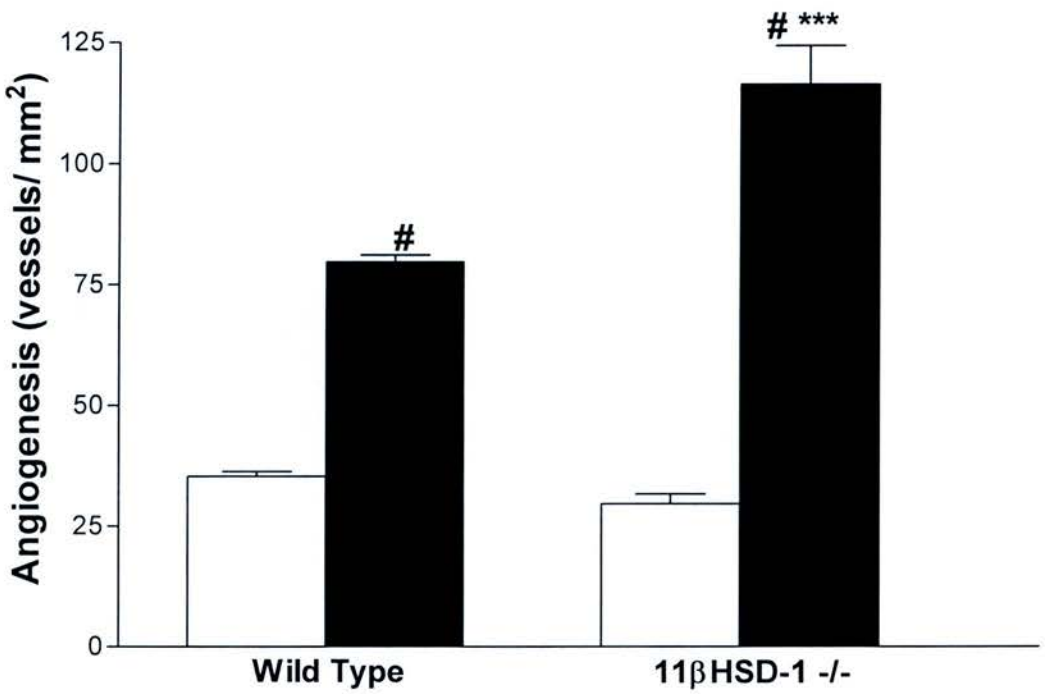


Figure 5.10 Myocardial angiogenesis following coronary artery ligation

Angiogenesis quantified as vessel/mm^2 assessed in Day 7 hearts from wild type and 11βHSD1 -/- mice. Ligation (filled columns) in C57Bl6 (wild type) and 11βHSD1 deficient (11βHSD1 -/-) mice increased angiogenesis in comparison to sham (open columns) (wild type n=6 sham, n=11 ligations and 11βHSD1 -/- n=5 sham, n=10 ligations). Results are mean ± SEM. # $p<0.001$ versus corresponding sham. *** $p<0.001$ for differences between wild type and 11βHSD1 -/-.

5.4 Discussion

Data presented in this chapter demonstrate that endogenous glucocorticoids inhibit angiogenesis in pathology in a glucocorticoid receptor dependent manner. Both myocardial revascularisation and cutaneous angiogenesis were increased in the presence of systemic anti-glucocorticoid therapy. In addition local glucocorticoid regeneration by 11βHSD1 regulated angiogenesis in these conditions. 11βHSD1 deficient mice exhibited increased revascularisation in cutaneous wounds and in the heart following myocardial infarction. Thus prevention of local regeneration of glucocorticoids produced an anti-glucocorticoid influence with similar effects on angiogenesis as systemic anti-glucocorticoid therapy. However, although both therapies were associated with improved revascularisation only 11βHSD1 deficiency was associated with a preservation of left ventricular function and parameters. How 11βHSD1 modulates left ventricular healing requires further evaluation but it appears that the improvements are not solely due to changes in revascularisation.

5.4.1 Assay development

Both a cutaneous and a myocardial angiogenesis model were used in these studies to allow the investigation of different tissues with distinct mechanisms of injury and dissimilar microenvironments for angiogenesis. Thus the results from these studies would be complimentary and may indicate important tissue and injury specific differences in angiogenesis regulation.

Choosing the time point post operatively to evaluate angiogenesis was important in these studies. Healing responses in both tissues are recognised to progress with time through inflammatory, proliferative and remodelling phases (Lutgens *et al.* 1999; Li *et al.* 2003; Virag & Murry 2003). Tissue revascularisation was expected to be

maximal at the end of proliferation and prior to remodelling with scar tissue formation (Singer & Clark 1999; Virag & Murry 2003). Appearance of new vessels with time in incised subcutaneous wounds in mice had been previously documented to be maximal at day 6 (Thompson *et al.* 1991) and the numbers of new vessels was not diminished by scar formation for up to 14 days (Galeano *et al.* 2003).

The appearance of new vessels with time following myocardial infarction in mice has not however been recorded previously. For although studies using genetically deficient mice have investigated effects on myocardial revascularisation, evaluation of angiogenesis has often been at an arbitrary time point and secondary to evaluations of structure, function infarct size or mortality (Lutgens *et al.* 1999; Carmeliet *et al.* 1996; Carmeliet & Collen 2000; Heymans *et al.* 1999). Therefore the current study recorded myocardial post-infarct angiogenesis with time and found revascularisation was well established by day 7. As a consequence of the sychronicity between the time of new vessel appearance in skin and heart, postoperative day 7 was chosen for tissue collection. Thus, mice undergoing coronary artery surgery provided both heart tissue and cutaneous thoracotomy wounds for angiogenesis study.

For both cutaneous and myocardial studies different techniques were available to initiate injury. Cutaneous wound healing has been evaluated following injury using sutured incision wounds (Thompson *et al.* 1991), or skin grafts (Gruss *et al.* 2003) or punch biopsy (Graiani *et al.* 2004). In this chapter cutaneous sutured incision wounds were used because of the established time scale of angiogenesis with this method (Thompson *et al.* 1991) and to make efficient use of animals undergoing surgery for coronary artery ligation. Similarly myocardial infarction can be induced by coronary

artery cauterisation or cryo-injury or ligation however ligation was chosen because it reliably creates an infarct of predictable size and causes minimal myocardial damage at application (Lutgens *et al.* 1999). Chronic ligation was adopted rather than an ischaemia/ reperfusion model because ischaemia/reperfusion models are associated with less predictable infarct sizes and inflammatory responses (Ytrehus & Downey 1993).

Identification of vessels in paraffin embedded sections from skin and heart required considerable method development. Vessels identified that were labelled by von Willebrand factor (vWF) appeared to be small to medium sized and not microvascular capillaries. Capillary detection may have been a more sensitive measure of angiogenesis since new vessel formation is acknowledged to be a microvascular response (Conway *et al.* 2001), however neither vWF nor CD31 antibody stains allowed detection of these vessels in the paraffin embedded sections. An explanation for these difficulties was published at completion of the surgeries and indicated that failure of these stains was probably as a result of the fixative used (Ismail *et al.* 2003). In this study 10% formalin was associated with poor antigen retrieval for most endothelial cell markers; in fact vWF was one of the few that produced any endothelial cell labelling with this fixative. Better results for endothelial cell antigen retrieval in mouse tissues were achieved with 1%paraformaldehyde or Zinc buffer fixatives (Ismail *et al.* 2003). Nevertheless in the studies presented in this chapter the numbers of vessels labelled with vWF and identified at x400 were consistent with previous reports of small to medium size vessel density at this magnification (Hao *et al.* 2004).

Infarct size was used in this chapter to evaluate the reliability and consistency of the injury to ensure all mice received similar infarcts. Infarct size measurement has been evaluated by several methods including assessment of haematoxylin and eosin stained sections (Virag & Murry 2003) but also by evaluating the size of infarct scar as indicated by collagen staining (Lutgens *et al.* 1999; Heymans *et al.* 1999). Collagen staining techniques were not used to evaluate infarcts in these studies because, although infarct size appears not to alter beyond a week (Lutgens *et al.* 1999), scar collagen deposition can continue to alter for up to 21 days (Lu *et al.* 2004; Yang *et al.* 2002). At these latter points infarct size measured by collagen can be used to determine differences in remodelling (Heymans *et al.* 1999) rather than consistency of surgery. Data from anatomical studies (Ahn *et al.* 2004; Yang *et al.* 2002; Lutgens *et al.* 1999) indicate that proximal ligation of the left main descending artery consistently induces infarction involving 40-45% of the left ventricle, thus the infarcts induced for the investigations of this chapter are consistent with established techniques.

Left ventricular remodelling occurs over 21 days in the murine model although the greatest changes occur over the first 7 days (Lutgens *et al.* 1999). The changes in left ventricular dimensions and function have been well characterised for C57Bl6 mice (Lutgens *et al.* 1999; Yang *et al.* 2002). A dramatic thinning of the left ventricular free wall occurs following infraction and is well established by day 7 (Lutgens *et al.* 1999). The loss of myocardium predisposes to ventricular rupture and left ventricular dysfunction. As a consequence of the loss of tissue and function the ventricle reshapes to accommodate the akinetic segment with hypertrophy of viable tissue and alteration of synchronicity of contraction (Lutgens *et al.* 1999; Yang *et al.* 2002).

Preservation of left ventricular function and cardiomyocyte mass is the function goal of revascularisation therapy (Michael *et al.* 1999). Prompt revascularisation is associated with preserved left ventricular dimensions and function (Pfeffer & Braunwald 1990). Therefore in these studies left ventricular dimensions and function were used as an evaluation of revascularisation. However this technique does not accurately quantify the functional ability of new vessels and techniques using fluorescent or radio labelled microspheres (van Royen *et al.* 2003) or magnetic resonance imaging (Streif *et al.* 2005) to detect collateral circulation would be required for this type of evaluation.

Similarly differences in cutaneous wound healing angiogenesis have been evaluated in several ways either by simple morphological analysis (Thompson *et al.* 1991), by examining tensile strength (Roman *et al.* 2002) or using laser doppler flow methods (Roman *et al.* 2002). Although tensile wound strength is affected by impaired angiogenesis in delayed wound healing (Li *et al.* 2003) whether enhanced angiogenesis improves wound strength is not clear (Roman *et al.* 2002) and therefore this technique was not adopted.

Laser Doppler assessment of flow was not available to measure collateral circulation in the healing skin and furthermore there were concerns regarding the use of this technique to assess capillary flow in the mice studied. It is possible that remaining wound sutures at 7 days may have created ultrasound shadows and distorted Doppler recordings. In addition the silk sutures may have contributed to an irritant effect that is known to cause an increased inter assay variation with this technique (Eun 1995). Therefore in this chapter wound angiogenesis was quantified by using morphological

analysis but also assessed by observing coat re-growth and tissue apposition and granulation.

Thus through the development of two different models performed by one procedure on the same animal, angiogenesis in a pathological setting could be examined in distinct yet linked environments. Both models were used to investigate the influence of glucocorticoids to regulate angiogenesis.

5.4.2 Application of models

RU38486 was used as a glucocorticoid receptor antagonist to investigate the influence of endogenous glucocorticoids on angiogenesis in pathology. Confirmation of systemic absorption of RU38486 was obtained by the measurement of plasma RU38486 and corticosterone concentrations. The range of RU38486 in mouse plasmas was lower that levels obtained clinically (Zhang *et al.* 1991) but is of the same order as that found in rodents given therapeutic doses (10mg/kg) of the drug (Heikinheimo *et al.* 1994).

Corticosterone plasma concentrations were elevated in both placebo and RU38486 treated animals as a consequence of sampling under stressful conditions following intraperitoneal injection and echocardiography under anaesthesia. However RU38486 treated animals had lower levels of plasma corticosterone than placebo controls. Whether this reflects inhibition of steroidogenic enzymes in the adrenal gland, an impaired stress response or partial agonist activity by RU38486 (Albertson *et al.* 1994; Schulz *et al.* 2002; Moldow *et al.* 2005) is unclear; but the findings in this chapter are consistent with those in chapter 4 that at this dose RU38486 reduces plasma corticosterone as measured by radioimmuno-assay.

Angiogenesis was enhanced in the presence of RU38486, indicating that endogenous glucocorticoids inhibit angiogenesis via a glucocorticoid receptor dependent mechanism. These data are consistent with results from other *in vivo* studies in Chapter 4 and also with previous reports of the effects of endogenous glucocorticoids on healing responses in rodents (Laue *et al.* 1988; Grose *et al.* 2002). However our current findings are novel in that they consider endogenous glucocorticoid effects on angiogenesis in the pathologies of cutaneous wounds and infarcted myocardium where new vessel formation is regulated by inflammation and ischaemia (Singer & Clark 1999; Heba *et al.* 2001).

In spite of improving angiogenesis RU38486 did not improve left ventricular remodelling or function. In addition there was a trend for an increase in the number of animals dying that had received RU38486 treatment, secondary to ventricular rupture, although the study was not powered to detect differences in mortality.

The trend for detrimental effects on mortality may be a reflection of the effects of systemic glucocorticoid antagonism to impair the stress response to myocardial infarction. In addition RU38486 may unbalance local glucocorticoid activities in the heart since glucocorticoids are ligands for mineralo- and glucocorticoid receptors both or which are found in cardiomyocytes (Escoubet *et al.* 1996; Sheppard 2003). Certainly over-expression of mineralocorticoid receptors in cardiomyocytes in rats is associated with detrimental effects on cardiomyocyte function (Le Menuet *et al.* 2004; Beggah *et al.* 2002). 11βHSD1, in contrast, is predicted to alter activation of any intracellular corticosteroid receptors, be they glucocorticoid or mineralocorticoid receptors. Whether selective glucocorticoid receptor antagonism in these cells by RU38486 is producing a similar effect by exposing the cells to unbalanced

glucocorticoid-mediated mineralocorticoid receptor activation will require further determination.

Other models have been used to examine the influence of therapeutic concentrations of glucocorticoids on myocardial ischaemia. The results from these studies are inconsistent, with some finding reduced infarct size (Hafezi-Moghadam *et al.* 2002; Kawabata *et al.* 1995; Valen *et al.* 2000) others demonstrating impaired healing (Scheuer & Mifflin 1997; Wexler 1976) and ventricular aneuryism (Vyden *et al.* 1974; Bulkley & Roberts 1974) . The variation in outcomes may represent the differences in species, dose of glucocorticoid, administrative protocols and mechanisms of infarct injury (LeGal & Morrissey 1990). Although mortality differences were not significant in the studies in this chapter the lack of improvement of function and remodelling despite improved revascularisation suggest that revascularisation alone is not sufficient to preserve cardiac function post myocardial infarction.

The angiogenic influence of a different anti-glucocorticoid approach was tried by using 11βHSD1 deficient transgenic mice. Prevention of local glucocorticoid regeneration by 11βHSD1 augmented the new vessel formation in ischaemic myocardium and injured skin and improved left ventricular function after infarction. 11βHSD1 is thus able to influence angiogenesis and modulate tissue repair in hypoxia and inflammation.

11βHSD1 activity was examined in homogenised heart to determine the effects of infarction on enzyme activity, since 11βHSD1 activity and expression is increased by inflammatory cytokines (Cai *et al.* 2001) and 11βHSD2 is regulated by hypoxia (Heiniger *et al.* 2003). Both dehydrogenase and reductase reactions were assayed

since although 11βHSD2 is an exclusive dehydrogenase, 11βHSD1 in homogenised tissue can exhibit both reductase and dehydrogenase activity. 11βHSD activity was not influenced by myocardial infarction: the absence of an effect may be as a consequence of cell loss or insufficient viable peri-infarct tissue or reflect the early time of tissue collection. 11βHSD1 has been documented in several cell types that would be present at different stages in the healing infarct, including mouse vascular smooth muscle cells (Christy *et al.* 2003), macrophages and rat cardiomyocytes (Sheppard & Autelitano 2002) and cardiac fibroblasts (Slight *et al.* 1993). The time of sampling may be critical in determining which cells are present for 11βHSD1 may well be differentially regulated in these different cell types. The absence of an effect may also be as a consequence of the tissue sampling technique. After bisection of hearts along the long axis and through the infarct half of the heart was utilised in the activity assay. Thus non-ischaemic un-infarcted left ventricle may have contributed to 11βHSD1 activity therefore created a false impression of activity in the infarct.

11βHSD1 activity in the skin has not previously been considered to influence wound healing in mice, although endogenous glucocorticoids are recognised to impair wound healing and adrenalectomy or RU38486 therapy has ameliorated wound repair in rodent models of impaired wound healing (Padgett *et al.* 1998; Glasper & DeVries 2005; Bitar *et al.* 1999). In other species 11βHSD1 is thought to influence inflammation in cutaneous tissues (Johnson *et al.* 2004). Whether absence of 11βHSD1 in vascular smooth muscle cells alone accounts for the enhance angiogenic responses observed in 11βHSD1 deficient mice is unclear. Certainly other cells within the cutaneous layer are acknowledged to express 11βHSD1 in other species including within the epidermis (Kenouch *et al.* 1994) or macrophages (Thieringer *et*

al. 2001) and it may be that local regulation of glucocorticoid concentration by these cells types also influences wound revascularisation.

Tensile strength of wounds was not measured as wound healing was not impaired in wild types. However, left ventricular function was used in the coronary artery ligation model to detect functional differences resulting from enhanced angiogenesis. 11βHSD1 deficiency in mice was associated with preserved left ventricular remodelling and preservation of left ventricular dimensions post myocardial infarction in comparison to wild type controls. It is unlikely that these responses are solely as a consequence of improved revascularisation, particularly in light of the results of treatment with RU38486. Instead remodelling may have been improved as a consequence of 11βHSD1 deficiency in inflammatory cells, cardiomyocytes or fibroblast (Christy *et al.* 2003; Sheppard & Autelitano 2002; Thieringer *et al.* 2001; Slight *et al.* 1993). 11βHSD1 transgenic deletion impairs macrophage phagocytic function that could prolong the acute inflammatory reaction or reduce the clearance of ailing cardiomyocytes (Chapman et al unpublished).

Other examples of 11βHSD1 deficiency protecting cells subjected to metabolic stress have been recorded, for example in neurons where 11βHSD1 deficiency reduced cognitive decline in aging mice (Yau *et al.* 2001) and inhibition of 11βHSD protects neurons in cell culture from chemical toxicity (Rajan *et al.* 1996). However contrary arguments that 11βHSD1 in fact safeguards tissues from excess inflammation are emerging from studies in the human ovary (Thomas *et al.* 1998; Rae *et al.* 2004; Tetsuka *et al.* 1999; Hillier & Tetsuka 1998). It is proposed that in the ovary 11βHSD1 up-regulation in follicular cells or surface epithelial cells at ovulation may repress peri-ovulatory inflammation in the corpus luteum. The significance of these

findings in mice is unclear since 11βHSD1 deficient mice are not infertile or susceptiblc to implantation failure as a result of luteal dysfunction (Kotelevtsev *et al.* 1997).

Alternatively the effects of 11βHSD1 transgenic deletion to improve left ventricular function may reflect metabolic consequences. 11βHSD1 deficient mice are noted to have differences in cellular and hepatic glucose metabolism that may be beneficial following myocardial infarction. 11βHSD1 deficient mice have reduced activation of hepatic gluconeogenesis enzymes in response to stress resulting in blunted hyperglycaemia (Kotelevtsev *et al.* 1997). Clinical studies improving glucose regulation post infarction have indicated that controlled glucose plasma concentrations are associated with improved mortality (Malmberg 1997).

The investigations in this chapter confirm that 11βHSD1 influences endogenous glucocorticoid regulation of angiogenesis, and have generated data that requires further evaluation in future studies. In myocardial infarction there is a striking effect of 11βHSD1 on left ventricular remodelling that was absent in RU38486 studies and appears not to be explained by revascularisation alone. Investigations to explore these differences are planned, particularly to examine the effect of combined mineralocorticoid and glucocorticoid antagonism on myocardial revascularisation and remodelling. Further investigations are planned to use the wound healing model to explore the role of 11βHSD1 in the integumentary system. Although gross observation did not detect any impairment of wound healing in C57BL6 mice, and thus the influence of 11βHSD1 was only apparent at histology, 11βHSD1 deficiency applied to a model of impaired wound healing (eg in Streptozocin induced diabetes

(Bitar *et al.* 1999) might further reveal the significance of 11βHSD1 in cutaneous tissues.

In summary in this chapter systemic anti-glucocorticoid therapy enhanced cutaneous and myocardial angiogenesis in pathology in a glucocorticoid receptor dependent manner. However, improved myocardial revascularisation was not associated with improved ventricular remodelling or function in these animals. In contrast in the presence of local anti-glucocorticoid effects in the 11βHSD-1 deficient mice, enhanced cutaneous and myocardial revascularisation was associated with preservation of left ventricular function and dimensions. The data on angiogenesis in pathology is consistent and complimentary with data in Chapters 3 and 4 indicating that endogenous glucocorticoids are sufficient to regulate angiogenesis modulated by the influence of 11βHSD1. The improvements in ventricular remodelling in 11βHSD1 deficient mice offers a novel approach to enhance myocardial revascularisation and preserve left ventricular function post myocardial infarction.

Chapter 6

Conclusions

Despite the extensive use of glucocorticoids, scientifically and clinically, to inhibit angiogenesis over the last 20 years, the influence of endogenous glucocorticoids on blood vessel growth has remained obscure. Furthermore, the ability of 11βHSDs to regulate new vessel formation by metabolism of glucocorticoids within the vessel wall has not been considered previously. Thus, the aim of the work described in this thesis was to determine whether metabolism of endogenous glucocorticoids in the vessel wall inhibits angiogenesis through the development and use of *in vitro*, *in vivo* and pathological models.

6.1 Materials and methods

Whether in aortic rings, subcutaneous sponge implant or wound healing models glucocorticoids at physiological concentrations inhibited angiogenesis. Three different models were used in order to demonstrate the constancy of the effect of glucocorticoids and to highlight the influence on angiogenesis of factors peculiar to each assay. The aortic ring model was used to examine the effects of glucocorticoids in the absence of systemic inflammation or ischaemia. The subcutaneous sponge implant assay was used to determine the angiostatic effects of endogenous glucocorticoids *in vivo* in the presence of an inflammatory response. Finally the subcutaneous injury and myocardial infarct models were used to assess the angiostatic effects of endogenous glucocorticoids in inflammation and ischaemia. By comparing the effects of glucocorticoids to influence angiogenesis in these three different models it is possible to draw some conclusions regarding the models themselves.

The aortic ring model was chosen from several available *in vitro* angiogenesis assays because it simulates most aspects of the angiogenic response and in comparison with other *in vitro* assays is perhaps most closely comparable to the situation *in vivo* (Carmeliet *et al.* 1998). Using this model angiogenesis was inhibited by physiologically relevant concentrations of glucocorticoids and these results were subsequently noted to be consistent through *in vivo* and *pathological* angiogenesis models. Thus the current findings confirm previous observations regarding the usefulness of the aortic ring model and indicate that this is a valuable tool to study angiogenesis.

The sponge implant model has been used as a wound healing assay to examine the effects of test substances on sponge collagenisation, fibrovascular infiltration and inflammatory cell invasion as measures of skin repair processes (Puolakkainen *et al.* 2005; Efron *et al.* 2001; Bradshaw *et al.* 2001). In this thesis both sponge implantation and cutaneous wound healing assays have been used. In comparing these models it could be concluded that the consistency of effect of glucocorticoids on angiogenesis in both indicates that the sponge model is a useful assay to examine angiogenesis in wound healing. However what has not been examined in this thesis are the other aspects of wound healing including inflammatory cell responses and scar formation with collagen deposition. In order to fully appreciate the strengths of the sponge implant model as a wound-healing assay these other aspects ought to be considered.

6.2 Inhibition of angiogenesis by glucocorticoids

Several different methods were used to investigate the influence of endogenous glucocorticoids. Physiologically relevant glucocorticoid concentrations were used *in vitro* whereas adrenalectomy and glucocorticoid receptor antagonism were used *in vivo*. In each case the influence of glucocorticoids at these concentrations was similar and always glucocorticoid receptor dependent. There was no deviation in the tissue specificity of the effect: cardiac, cutaneous and aortic tissues were all sensitive to the angiostatic effects of glucocorticoids and uniformly it was the glucocorticoid receptor that was responsible for these effects across these different tissues.

6.3 Influence of 11βHSD-1 on glucocorticoid induced angiostasis

Consistently through the models studied inhibition of 11βHSD-1 enhanced angiogenesis. 11βHSD-1 therefore impairs angiogenesis in aortic, cutaneous and myocardial tissue. Although 11βHSD-1 is expressed in mouse aortic tissue (Christy *et al.* 2003) and in this thesis we observed 11βHSD reductase activity in aortic and heart tissue it may not be expressed across all vascular beds or tissues. It is unclear whether 11βHSD-1 regulates angiogenesis in tissues where there is considerable expression of 11βHSD-2. In the kidney for example where 11βHSD-2 is highly expressed by the distal convoluted tubules, angiogenesis control by 11βHSD-1 may be less significant.

Nevertheless in aortic tissue we did consider the effect of 11βHSD-2 by examining the action of the non-selective 11βHSD inhibitor carbenoxolone on the influence of

corticosterone. The absence of an effect suggested that in aortic tissue *in vitro* 11βHSD-2 does not influence angiogenesis. Whether 11βHSD-2 *in vivo* could directly influence angiogenesis in a tissue specific manner for example in the kidneys may be difficult to dissect from the effects of 11βHSD-2 on hypertension and vascular function.

6.4 Potential clinical application of the results

Angiogenesis is an important determinant in many diseases: in cancer aberrant angiogenesis regulation contributes to tumour size and metastases (Garcea *et al.* 2004). In diabetes mellitus impaired cutaneous revascularisation impairs healing and leads to the formation of cutaneous ulcers (Galiano *et al.* 2004). In contrast in the retina of diabetic patients angiogenesis produces proliferative diabetic retinopathy and may lead to blindness. In cardiovascular disease revascularisation remains a therapeutic target not just in myocardial ischaemia but also in stroke and peripheral vascular disease. How might the findings from this thesis be projected into clinical applications?

Clinically glucocorticoids are already employed in the treatment of vascular tumours to inhibit angiogenesis (Hasan *et al.* 2000). The data in this thesis suggest that the effects of glucocorticoids on angiogenesis in these tumours are glucocorticoid receptor dependent.

There may be other indications for glucocorticoids to treat tumours in situations where tumour shrinkage is required, or if a decrease in tumour vascularity desirable. This would be the case for example prior to debulking procedures or surgical resection. Conceivably there might be a role for glucocorticoids to be used following

such procedures to reduce metastatic growth. However there could be problems with wound healing post surgery with systemic glucocorticoid therapy.

Whether all tumours are susceptible to the effects of glucocorticoids is unclear and tissue specific expression of 11βHSDs could be significant in determining tumour sensitivity to glucocorticoid therapy. For example since 11βHSD2 is highly expressed in the colon (Takahashi *et al.* 1998) and inactivates glucocorticoids glucocorticoid-regulation of angiogenic processes in colonic cancer may be significantly reduced. There is some circumstantial evidence for this type of effect in that expression of 11βHSD2 in ACTH producing small cell lung carcinomas is thought to contribute their glucocorticoid-resistance phenotype (Parks *et al.* 1998). Not withstanding these arguments however, there is often dysregulated gene expression within tumours that may alter the tissue specific expression of 11βHSDs to change the susceptibility to glucocorticoid angiostasis.

In fact in many tumours there is absent or low expression of 11βHSD-1 but high expression of 11βHSD-2 [see table 6.1 (Rabbitt *et al.* 2003)]. This may represent a change in gene expression associated with de-differentiation or could be a primary event in tumour development contributing to cell proliferation and the persistence of undifferentiated cells. In either case these observations are not in disagreement with our current findings that suggest if a tumour down regulated 11βHSD-1; angiogenesis would be enhanced. Thus tumour cells with such expression would be selected, as unregulated angiogenesis in this setting would be advantageous for tumour growth and haematogenous metastatic spread.

The observed trend for expression of 11βHSD-2 and reduced or absent expression of 11βHSD-1in tumours (see table 6.1) suggests that many cancers will be resistant to

the angiostatic effects of glucocorticoids. Whether 11βHSD- 2 specific inhibitors or gene therapy with 11βHSD-1 could alter this phenotype remains unclear. The data in this thesis compliments the impression that is evolving with regard to the effects of 11βHSD-1 as a differentiation inducing, anti-proliferative and now an anti-angiogenic factor. If it were possible to harness these features of 11βHSD-1 activity, 11βHSD-1 would make a potential target for chemotherapy.

Table 6.1

Study Model	11βHSD1 expression	11βHSD2 expression
Osteosarcoma cell lines (TE-85, MG-63, SaOS-2)	-	+
Fibrosarcoma cell line (Hs913)	-	+
Endometrial cancer cell line (Ishikawa)	-	+
Breast Cancer cell lines (MCF-7,ZR75-1, PMC42)	-	+
Myelomonocytic cell line (U937)	-	+
Colon carcinoma	-	+
Colon cancer cell lines (Caco-2, Ht-29)	-	+
Breast tumour specimens	-	+
Pituitary ademoma	-	+
Squamous cell carcimona of the head and neck	↓	

Table 6.1 Summary of 11βHSD 1 isozyme expression in neoplastic tissues and cell lines (Rabbitt *et al.* 2003)

(-) Represents no detectable expression (+) indicates expression is present (↓) denotes reduced expression.

In diabetes mellitus and the metabolic syndrome 11βHSD-1 expression and activity in adipose tissue has been identified as a potential therapeutic target. In obese humans and rodents there is increased expression of 11βHSD-1 in adipose tissue (Wake *et al.* 2003; Masuzaki *et al.* 2001). This increased expression appears to be of pathogenic importance because transgenic mice over-expressing 11βHSD-1 selectively in adipose tissue exhibit a full metabolic syndrome with visceral obesity, dyslipidaemia, insulin-resistant diabetes, and hypertension (Masuzaki *et al.* 2001). Although 11βHSD-1 inhibition might be attractive to improve metabolic parameters in diabetes the data in this thesis suggest that 11βHSD-1 inhibition could be contra-indicated in some diabetics for it may enhance angiogenesis in patients at risk of proliferative diabetic retinopathy. Whether specific vascular beds could be targeted with 11βHSD-1 inhibitors might be a possibility with gene therapy. Viral vectors to deliver gene therapy are being designed to recognise endothelial peptides that identify specific vascular beds (Trepel *et al.* 2002; Kolonin *et al.* 2001). Thus in diabetics targeted therapy could deliver 11βHSD-1 anti-sense gene therapy to adipose tissue vascular beds to improve metabolic indices in diabetes. In addition in patients with diabetic ulcers 11βHSD-1 anti-sense gene therapy directed to the cutaneous vasculature may ameliorate ulcer healing by enhancing angiogenesis. Thus the findings in this thesis, although adding to the complexity of the influence of 11βHSD-1 in diabetes, have revealed a potential harmful side effect of 11βHSD1 inhibitor therapy. Therefore upon identification of specific 11βHSD-1 inhibitors in the future; careful screening and subsequent follow up of potential clinical trial patients would need to be undertaken to identify those at risk of diabetic retinopathy.

Glucocorticoid receptor antagonism or 11βHSD-1 deficiency improved myocardial revascularisation following coronary artery ligation. Improved revascularisation was not however solely responsible for preserved left ventricular function: for the ventricular remodelling outcomes in 11βHSD-1 -/- mice in comparison to RU38486 treated mice were strikingly different. The preservation of left ventricular function following coronary artery ligation in 11βHSD-1 -/- mice requires further investigation but may reflect differences in cellular immunity, or altered glucocorticoid activity at mineralocorticoid receptors in cardiomyocytes. Clinically mineralocorticoid receptor antagonist have been introduced for the treatment of ventricular dysfunction in acute myocardial infarction following the success of a recent clinical trial (Pitt *et al.* 2005). Despite the success of this therapy the mechanisms for its benefit remain obscure. The endogenous ligand acting at the mineralocorticoid receptor in cardiac tissue is yet to be determined. The greater intra-cellular availability of glucocorticoids in comparison to mineralocorticoids and in the absence of significant cardiac expression of 11βHSD-2 suggests that the active ligands are glucocorticoids. Thus 11βHSD-1 may present a novel target to improve myocardial revascularisation and preserve left ventricular function following myocardial infarction.

6.5 Summary

In summary, the research present in this thesis demonstrates that endogenous glucocorticoids regulated by 11βHSD1 are sufficient to inhibit angiogenesis *in vitro*, *in vivo* and *in pathology* in inflammatory and ischaemic conditions. The findings of these studies propose a novel role for vascular 11βHSD1 in the regulation of angiogenesis. In addition, the influence of 11βHSD1 to modulate tissue repair as well as revascularisation following infarction suggests that the function of 11βHSD1 in repairing tissues maybe more complex than first appreciated.

6.6 Future Studies

The studies presented in this thesis have provided a basis for future work aimed at the elucidation of mechanisms for glucocorticoid-induced angiostasis and further explanation of the influence of 11βHSD1 on tissue remodelling

Mechanism- Plans to explore the mechanism of glucocorticoid actions are being developed with the framework that glucocorticoids may influence proliferative and mitotic factors, cell migration, inflammation or the extracellular matrix.

Proliferative and mitotic factors- It is proposed to use the aortic ring model of angiogenesis to investigate potential interactions with glucocorticoids and key mediators of angiogenesis, such as VEGF and nitric oxide. Adaptation of this model to use collagen based gels in order to facilitate cell recovery and subsequent RNA extraction will aid these studies.

The pilot HUVEC data is to be extended to examine the influence of glucocorticoids on intracellular calcium transients and begin to elucidate how glucocorticoids may

disrupt the entry of extracellular calcium. Other sources of endothelial cells could be used for this work although previous attempts to obtain primary cultures of mouse aortic endothelial cells have been difficult. One possible approach is to use sponge implants as a source of mouse endothelial cells which could be exploited to provide primary cultures with which to further examine the effect of glucocorticoids on VEGF signalling.

Cell migration- Although primary mouse endothelial cell cultures are not currently available the influence of glucocorticoids on HUVEC migration is being investigated as part of a new study to determine the mechanism of glucocorticoid-induced angiostasis.

Inflammation- A reason for the initial proposals to concentrate on *in vitro* studies using endothelial cells has been to remove systemic and local inflammatory influences from angiogenesis conditions and, therefore, explore whether glucocorticoids have direct angiostatic effects.

Extracellular Matrix- It is aimed to investigate interactions between glucocorticoids and matrix metalloproteinases using initially the aortic ring assay but also in the chronic coronary artery ligation model.

Tissue Remodelling- Further investigation is proposed to determined the causes of the dramatic difference between systemic glucocorticoid antagonism and the effects of 11βHSD1 deficiency on left ventricular remodelling post infarction. The preservation of left ventricular function with 11βHSD1 deficiency requires further evaluation to dissect out the contributions from possible interactions with angiogenesis, inflammation and matrix remodelling.

Furthermore it is hoped that with the successful development of selective 11βHSD1 inhibitors that are being formulated by biotech and pharmaceutical companies, 11βHSD1 inhibition could be evaluated as a potential cardiovascular therapy post myocardial infarction.

Bibliography

Addison T 1855 *On the constitutional and local effects of disease of the supra-renal capsules*. London: reprinted by S Highley.

Agarwal AK, Monder C, Eckstein B & White PC 1989 Cloning and expression of rat cDNA encoding corticosteroid 11β-dehydrogenase. *Journal of Biological Chemistry* 264 18939-18943.

Agarwal AK, Mune T, Monder C & White PC 1994 NAD^+-dependent isoform of 11β-hydroxysteroid dehydrogenase. Cloning and characterisation of cDNA from sheep kidney. *Journal of Biological Chemistry* 269 25959-25962.

Agarwal AK, Tusie-Luna M-T, Monder C & White PC 1990 Expression of 11β-hydroxysteroid dehydrogenase using recombinant vaccinia virus. *Molecular Endocrinology* 4 1827-1832.

Aguilera G, Rabadan-Diehl C & Nikodemova M 2001 Regulation of pituitary corticotropin releasing hormone receptors. *Peptides* 22 769-774.

Ahn D, Cheng L, Moon C, Spurgeon H, Lakatta EG & Talan MI 2004 Induction of myocardial infarcts of a predictable size and location by branch pattern probability-assisted coronary ligation in C57BL/6 mice. *American Journal of Physiology- Heart and Circulatory Physiology* 286 H1201-H1207.

Ahtikoski AM, Riso EM, Koskinen SO, Risteli J & Takala TE 2004 Regulation of type IV collagen gene expression and degradation in fast and slow muscles during dexamethasone treatment and exercise. *Pflügers Archiv European Journal of Physiology.* 448 123-130.

Akita M, Murata E, Merker HJ & Kaneko K 1997 Formation of new capillary-like tubes in a three-dimensional in vitro model (aorta/collagen gel). *Anatomischer Anzieger.* 179 137-147.

Albertson BD, Hill RB, Sprague KA, Wood KE, Nieman LK & Loriaux DL 1994 Effect of the antiglucocorticoid RU486 on adrenal steroidogenic enzyme activity and steroidogenesis. *European Journal of Endocrinology.* 130 195-200.

Albiston AL, Obeyesekere VR, Smith RE & Krozowski ZS 1994 Cloning and tissue distribution of the human 11β-hydroxysteroid dehydrogenase type 2 enzyme. *Molecular and Cellular Endocrinology* 105 R11-R17.

Alderton WK, Cooper CE & Knowles RG 2001 Nitric oxide synthases: structure, function and inhibition. *Biochemical Journal* 357 593-615.

Alikhani-Koopaei R, Fouladkou F, Frey FJ & Frey BM 2004 Epigenetic regulation of 11 beta-hydroxysteroid dehydrogenase type 2 expression. *Journal of Clinical Investigation* 114 1146-1157.

Almawi WY, Abou Jaoude MM & Li XC 2002 Transcriptional and post-transcriptional mechanisms of glucocorticoid antiproliferative effects. *Hematology and Oncology.* 20 17-32.

Amanatullah DF, Zafonte BT & Pestell RG 2002 The cell cycle in steroid hormone regulated proliferation and differentiation. *Minerva Endocrinology.* 27 7-20.

Amelung D, Huebner HJ, Roka L & Meyerheim G 1953 Conversion of cortisone to compound F. *Journal of Clinical Endocrinology and Metabolism* 13 1125.

Andrade SP, Fan TP & Lewis GP 1987b Quantitative in-vivo studies on angiogenesis in a rat sponge model. *British Journal of Experimental Pathology.* 68 755-766.

Aranyi P & Machovich R 1985 Glucocorticoid receptor is activated by heparin and deactivated by plasmin. *Acta Biochimica Biophysica Academiae Scientiarum Hungaricae.* 20 129-133.

Arriza JL, Weinberger C & Cerelli G 1987 Cloning of human mineralocorticoid receptor complementary DNA; structural and functional kinship with the glucocorticoid receptor. *Science* 237 268-275.

Auerbach R, Lewis R, Shinners B, Kubai L & Akhtar N 2003b Angiogenesis assays: a critical overview. *Clinical Chemistry* 49 32-40.

Babaei S, Teichert-Kuliszewska K, Monge JC, Mohamed F, Bendeck MP & Stewart DJ 1998 Role of nitric oxide in the angiogenic response in vitro to basic fibroblast growth factor. *Circulation Research* 82 1007-1015.

Bader BL, Rayburn H, Crowley D & Hynes RO 1998 Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. *Cell* 95 507-519.

Barnes PJ 1998 Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clinical Science (London)* 94 557-572.

Bator JM, Cohen RL & Chambers DA 1998 Hydrocortisone regulates the dynamics of plasminogen activator and plasminogen activator inhibitor expression in cultured murine keratinocytes. *Experimental Cell Research.* 242 110-119.

Beer HD, Fassler R & Werner S 2000 Glucocorticoid-regulated gene expression during cutaneous wound repair. *Vitamins and Hormones.* 59 217-239.

Beggah AT, Escoubet B, Puttini S, Cailmail S, Delage V, Ouvrard-Pascaud A, Bocchi B, Peuchmaur M, Delcayre C, Farman N & Jaisser F 2002 Reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes. *Proceedings of the National Academy of Sciences U.S.A* 99 7160-7165.

Bellon G, Martiny L & Robinet A 2004 Matrix metalloproteinases and matrikines in angiogenesis. *Critical Reviews in Oncology and Hematology.* 49 203-220.

Benediktsson R, Lindsay RS, Noble J, Seckl JR & Edwards CRW 1993 Glucocorticoid exposure in utero: new model for adult hypertension. *Lancet* 341 339-341.

Berk BC, Gordon JB & Alexander RW 1991 Pharmacologic roles of heparin and glucocorticoids to prevent restenosis after coronary angioplasty. *Journal of the American College of Cardiology* 17 111B-117B.

Bernini GP, Moretti A, Bonadio AG, Menicagli M, Viacava P, Naccarato AG, Iacconi P, Miccoli P & Salvetti A 2002 Angiogenesis in human normal and pathologic adrenal cortex. *Journal of Clinical Endocrinology and Metabolism* 87 4961-4965.

Berridge MJ 1987 Inositol trisphosphate as a second messenger in signal transduction. *Annals New York Academy of Sciences* 494 39-51.

Beuschlein F, Fassnacht M, Klink A, Allolio B & Reincke M 2001 ACTH-receptor expression, regulation and role in adrenocortial tumor formation. *European Journal of Endocrinology.* 144 199-206.

Biancone L, Martino AD, Orlandi V, Conaldi PG, Toniolo A & Camussi G 1997 Development of inflammatory angiogenesis by local stimulation of Fas in vivo. *Journal of Experimental Medicine.* 186 147-152.

Bitar MS, Farook T, Wahid S & Francis IM 1999 Glucocorticoid-dependent impairment of wound healing in experimental diabetes: amelioration by adrenalectomy and RU 486. *Journal of Surgical Research.* 82 234-243.

Blei F, Wilson EL, Mignatti P & Rifkin DB 1993 Mechanism of action of angiostatic steroids: suppression of plasminogen activator activity via stimulation of plasminogen activator inhibitor synthesis. *Journal of Cellular Physiology* 155 568-578.

Blomme EA, Chinn KS, Hardy MM, Casler JJ, Kim SH, Opsahl AC, Hall WA, Trajkovic D, Khan KN & Tripp CS 2003 Selective cyclooxygenase-2 inhibition does not affect the healing of cutaneous full-thickness incisional wounds in SKH-1 mice. *British Journal of Dermatology* 148 211-223.

Borst JGG, Ten Holt SP & DeVries LA 1953 Synergistic action of liquorice and cortisone in Addison's and Simmond's disease. *Lancet* i 657-663.

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry.* 72 248-254.

Bradshaw AD, Reed MJ, Carbon JG, Pinney E, Brekken RA & Sage EH 2001 Increased fibrovascular invasion of subcutaneous polyvinyl alcohol sponges in SPARC-null mice. *Wound Repair and Regeneration.* 9 522-530.

Brandt PC & Vanaman TC 2000 Elevated glucocorticoid receptor transactivation and down-regulation of alpha 1 integrin are associated with loss of plasma membrane Ca2+-ATPase isoform 1. *Journal of Biological Chemistry* 275 24534-24539.

Brem AS, Bina RB, Hill N, Alia C & Morris DJ 1997 Effects of licorice derivatives on vascular smooth muscle function. *Life Sciences* 60 207-214.

Brem AS, Bina RB, King T & Morris DJ 1995 Bidirectional activity of 11β-hydroxysteroid dehydrogenase in vascular smooth muscle cells. *Steroids* 60 406-410.

Brem AS, Bina RB, King TC & Morris DJ 1998 Localization of 2 11beta-OH steroid dehydrogenase isoforms in aortic endothelial cells. *Hypertension* 31 459-462.

Brereton PS, van Driel RR, Suhaimi F, Koyama K, Dilley R & Krozowski Z 2001 Light and electron microscopy localization of the 11beta-hydroxysteroid dehydrogenase type I enzyme in the rat. *Endocrinology* 142 1644-1651.

Brown EB, Campbell RB, Tsuzuki Y, Xu L, Carmeliet P, Fukumura D & Jain RK 2001 In vivo measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. *Nature.Medicine.* 7 864-868.

Brown RW, Chapman KE, Edwards CRW & Seckl JR 1993 Human placental 11β-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinology* 132 2614-2621.

Brown RW, Chapman KE, Koteletsev Y, Yau JL, Lindsay RS, Brett LP, Leckie CM, Murad P, Lyons V, Mullins JJ, Edwards CRW & Seckl JR 1996a Cloning and production of antisera to human placental 11β-hydroxysteroid dehydrogenase type 2. *Biochemical Journal* 313 1007-1017.

Brown RW, Diaz R, Robson AC, Kotelevtsev YV, Mullins JJ, Kaufman MH & Seckl JR 1996b The ontogeny of 11beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. *Endocrinology* 137 794-797.

Bujalska IJ, Kumar S, Hewison M & Stewart PM 1999 Differentiation of adipose stromal cells: The roles of glucocorticoids and 11beta-hydroxysteroid dehydrogenase. *Endocrinology* 140 3188-3196.

Bujalska IJ, Walker EA, Tomlinson JW, Hewison M & Stewart PM 2002 11Beta-hydroxysteroid dehydrogenase type 1 in differentiating omental human preadipocytes: from de-activation to generation of cortisol. *Endocrine Research.* 28 449-461.

Bulkley BH & Roberts WC 1974 Steroid therapy during acute myocardial infarction. A cause of delayed healing and of ventricular aneurysm. *American Journal of Medicine* 56 244-250.

Buttgereit F 2000 Mechanisms and clinical relevance of nongenomic glucocorticoid actions. *Zeitschrift für Rheumatologie.* 59 Suppl 2 II/119-II/123.

Byrne JD, Wing DA, Fraser M, Fassett MJ, Goodwin TM & Challis JR 2004 Mifepristone: effect on plasma corticotropin-releasing hormone, adrenocorticotropic hormone, and cortisol in term pregnancy. *Journal of Perinatology.* 24 416-420.

Cai TQ, Wong BM, Mundt SS, Thieringer R, Wright SD & Hermanowski-Vosatka A 2001 Induction of 11beta-hydroxysteroid dehydrogenase type 1 but not type 2 in human aortic smooth muscle cells by inflammatory stimuli. *Journal of Steroid Biochemistry* 77 117-122.

Campochiaro PA, Soloway P, Ryan SJ & Miller JW 1999 The pathogenesis of choroidal neovascularization in patients with age-related macular degeneration. *Molecular Vision.* 5 34.

Cariou R, Harousseau JL & Tobelem G 1988 Inhibition of human endothelial cell proliferation by heparin and steroids. *Cell biology international reports.* 12 1037-1047.

Carmeliet P 2000 Mechanisms of angiogenesis and arteriogenesis. *Nature Medicine.* 6 389-395.

Carmeliet P & Collen D 2000 Transgenic mouse models in angiogenesis and cardiovascular disease. *Journal of Pathology.* 190 387-405.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W & Nagy A 1996 Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380 435-439.

Carmeliet P & Jain RK 2000 Angiogenesis in cancer and other diseases. *Nature* 407 249-257.

Carmeliet P, Moons L & Collen D 1998 Mouse models of angiogenesis, arterial stenosis, atherosclerosis and hemostasis. *Cardiovascular Research* 39 8-33.

Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D & Persico MG 2001 Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nature Medicine.* 7 575-583.

Casonato A, Pontara E, Boscaro M, Sonino N, Sartorello F, Ferasin S & Girolami A 1999 Abnormalities of von Willebrand factor are also part of the prothrombotic state of Cushing's syndrome. *Blood Coagulation and Fibrinolysis* 10 145-151.

Cheng SL, Lai CF, Fausto A, Chellaiah M, Feng X, McHugh KP, Teitelbaum SL, Civitelli R, Hruska KA, Ross FP & Avioli LV 2000 Regulation of alphaVbeta3 and alphaVbeta5 integrins by dexamethasone in normal human osteoblastic cells. *Journal of Cellular Biochemistry.* 77 265-276.

Christy C, Hadoke PWF, Paterson JM, Mullins JJ, Seckl JR & Walker BR 2003 Glucocorticoid action in mouse aorta; localisation of 11beta Hydroxysteroid dehydrogenase type 2 and effects on response to glucocorticoid in vitro. *Hypertension* 42 580-587.

Ciulla MM, Paliotti R, Ferrero S, Braidotti P, Esposito A, Gianelli U, Busca G, Cioffi U, Bulfamante G & Magrini F 2004 Left ventricular remodeling after experimental myocardial cryoinjury in rats. *Journal of Surgical Research.* 116 91-97.

Clark AJ, Baig AH, Noon L, Swords FM, Hunyady L & King PJ 2003 Expression, desensitization, and internalization of the ACTH receptor (MC2R). *Annals New York Academy of Sciences* 994 111-117.

Cleasby ME, Livingstone DE, Nyirenda MJ, Seckl JR & Walker BR 2003 Is programming of glucocorticoid receptor expression by prenatal dexamethasone in the rat secondary to metabolic derangement in adulthood? *European Journal of Endocrinology.* 148 129-138.

Collinson DJ & Donnelly R 2004 Therapeutic angiogenesis in peripheral arterial disease: can biotechnology produce an effective collateral circulation? *European .Journal of Vascular and Endovascular Surgery.* 28 9-23.

Colville-Nash PR & Scott DL 1992 Angiogenesis and rheumatoid arthritis: pathogenic and therapeutic implications. *Annals of Rheumatic Diseases.* 51 919-925.

Conway EM, Collen D & Carmeliet P 2001 Molecular mechanisms of blood vessel growth. *Cardiovascular Research* 49 507-521.

Cooper MS, Walker EA, Bland R, Fraser WD, Hewison M & Stewart PM 2000 Expression and functional consequences of 11beta-hydroxysteroid dehydrogenase activity in human bone. *Bone* 27 375-381.

Crum R, Szabo S & Folkman J 1985 A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science* 230 1375-1378.

Cunningham SA, Tran TM, Arrate MP, Bjercke R & Brock TA 1999 KDR activation is crucial for VEGF165-mediated Ca2+ mobilization in human umbilical vein endothelial cells. *American Journal of Physiology* 276 C176-C181.

Cushing H 1912 *The pituitary body and its disorders*. Philadelphia and London: Lippincott.

Datta YH, Romano M, Jacobson BC, Golan DE, Serhan CN & Ewenstein BM 1995 Peptido-leukotrienes are potent agonists of von Willebrand factor secretion and P-

selectin surface expression in human umbilical vein endothelial cells. *Circulation* 92 3304-3311.

Dellian M, Witwer BP, Salehi HA, Yuan F & Jain RK 1996 Quantitation and physiological characterization of angiogenic vessels in mice: effect of basic fibroblast growth factor, vascular endothelial growth factor/vascular permeability factor, and host microenvironment. *American Journal of Pathology.* 149 59-71.

Derbyshire EJ, Yang YC, Li S, Comin GA, Belloir J & Thorpe PE 1996 Heparin-steroid conjugates lacking glucocorticoid or mineralocorticoid activities inhibit the proliferation of vascular endothelial cells. *Biochimica Biophysica Acta* 1310 86-96.

Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S & Akhurst RJ 1995 Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* 121 1845-1854.

Doll R, Langman MJ & Shawdon HH 1968 Treatment of gastric ulcer with carbenoxolone: antagonistic effect of spironolactone. *Gut* 9 42-45.

Dormond O, Foletti A, Paroz C & Ruegg C 2001 NSAIDs inhibit alpha V beta 3 integrin-mediated and Cdc42/Rac-dependent endothelial-cell spreading, migration and angiogenesis. *Nature Medicine.* 7 1041-1047.

Draper N, Walker EA, Bujalska IJ, Tomlinson JW, Chalder SM, Arlt W, Lavery GG, Bedendo O, Ray DW, Laing I, Malunowicz E, White PC, Hewison M, Mason PJ, Connell JM, Shackleton CH & Stewart PM 2003 Mutations in the genes encoding 11beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase interact to cause cortisone reductase deficiency. *Nature.Genetics.* 34 434-439.

Eden S 1979 Age- and sex-related differences in episodic growth hormone secretion in the rat. *Endocrinology* 105 555-560.

Edwards RH, Sarmenta SS & Hass GM 1960 Stimulation of granulation tissue growth by tissue extracts. Study in intramuscular wounds in rabbits. *Archives in Pathology.* 69 286-302.

Efron DT, Most D, Shi HP, Tantry US & Barbul A 2001 A novel method of studying wound healing. *Journal of Surgical Research.* 98 16-20.

Epstein MT, Espiner EA, Donald RA & Hughes H 1977 Effect of eating liquorice on the renin-angiotensin-aldosterone axis in normal subjects. *British Medical Journal* 6059 488-490.

Escher G, Galli I, Vishwanath BS, Frey BM & Frey FJ 1997 Tumor necrosis factor alpha and interleukin 1beta enhance the cortisone/cortisol shuttle. *Journal of Experimental Medicine* 186 189-198.

Escher G, Nawrocki A, Staub T, Vishwanath BS, Frey BM, Reichen J & Frey FJ 1998 Down-regulation of hepatic and renal 11beta-hydroxysteroid dehydrogenase in rats with liver cirrhosis. *Gastroenterology* 114 175-184.

Escoubet B, Coureau C, Blot-Chabaud M, Bonvalet JP & Farman N 1996 Corticosteroid receptor mRNA expression is unaffected by corticosteroids in rat kidney, heart, and colon. *American Journal of Physiology* 270 C1343-C1353.

Eun HC 1995 Evaluation of skin blood flow by laser Doppler flowmetry. *Clinical Dermatology.* 13 337-347.

Evagelatou M, Peterson SL & Cooke BA 1997 Leukocytes modulate 11beta-hydroxysteroid dehydrogenase (11beta-HSD) activity in human granulosa-lutein cell cultures. *Molecular & Cellular Endocrinology* 133 81-88.

Faehling M, Kroll J, Fohr KJ, Fellbrich G, Mayr U, Trischler G & Waltenberger J 2002b Essential role of calcium in vascular endothelial growth factor A-induced signaling: mechanism of the antiangiogenic effect of carboxyamidotriazole. *Federation of American Societies for Experimental Biology Journal* 16 1805-1807.

Fajardo LF, Kwan HH, Kowalski J, Prionas SD & Allison AC 1992 Dual role of tumor necrosis factor-alpha in angiogenesis. *American Journal of Pathology.* 140 539-544.

Fakih M, Johnson CS & Trump DL 2002 Glucocorticoids and treatment of prostate cancer: a preclinical and clinical review. *Urology* 60 553-561.

Finkenzeller G, Technau A & Marme D 1995 Hypoxia-induced transcription of the vascular endothelial growth factor gene is independent of functional AP-1 transcription factor. *Biochemical and Biophysical Research Communications* 208 432-439.

Fischer S, Renz D, Schaper W & Karliczek GF 2001 In vitro effects of dexamethasone on hypoxia-induced hyperpermeability and expression of vascular endothelial growth factor. *European Journal of Pharmacology* 411 231-243.

Flugel-Koch C, Ohlmann A, Fuchshofer R, Welge-Lussen U & Tamm ER 2004 Thrombospondin-1 in the trabecular meshwork: localization in normal and glaucomatous eyes, and induction by TGF-beta1 and dexamethasone in vitro. *Experimental Eye Research.* 79 649-663.

Folkman J 2001 Angiogenesis. In *Principles of Internal Medicine*, edn 15th, pp 517-530. Eds E Braunwald, SL Hauser, AC Fauci, DL Longo, Jameson J.L. & Kasper D.L. New York: McGraw-Hill.

Folkman J & Ingber DE 1987 Angiostatic steroids. Method of discovery and mechanism of action. *Annals of Surgery* 206 374-383.

Folkman J, Langer R, Linhardt RJ, Haudenschild C & Taylor S 1983 Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. *Science* 221 719-725.

Folkman J & Shing Y 1992 Angiogenesis. *Journal of Biological Chemistry* 267 10931-10934.

Fox SB, Leek RD, Weekes MP, Whitehouse RM, Gatter KC & Harris AL 1995 Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count, and computer image analysis. *Journal of Pathology.* 177 275-283.

Frankfurt O & Rosen ST 2004 Mechanisms of glucocorticoid-induced apoptosis in hematologic malignancies: updates. *Current Opinion in Oncology.* 16 553-563.

Fukumura D & Jain RK 1998 Role of nitric oxide in angiogenesis and microcirculation in tumors. *Cancer Metastasis Reviews.* 17 77-89.

Funder JW, Pearce PT, Smith R & Campbell J 1989 Vascular type 1 aldosterone binding sites are physiological mineralocorticoid receptors. *Endocrinology* 125 2224-2226.

Galeano M, Deodato B, Altavilla D, Cucinotta D, Arsic N, Marini H, Torre V, Giacca M & Squadrito F 2003 Adeno-associated viral vector-mediated human vascular endothelial growth factor gene transfer stimulates angiogenesis and wound healing in the genetically diabetic mouse. *Diabetologia* 46 546-555.

Galiano RD, Tepper OM, Pelo CR, Bhatt KA, Callaghan M, Bastidas N, Bunting S, Steinmetz HG & Gurtner GC 2004 Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. *American Journal of Pathology.* 164 1935-1947.

Gametchu B, Chen F, Sackey F, Powell C & Watson CS 1999 Plasma membrane-resident glucocorticoid receptors in rodent lymphoma and human leukemia models. *Steroids* 64 107-119.

Gao H-B, Ge R-S, Lakshmi V, Marandici A & Hardy MP 1997 Hormonal regulation of oxidative and reductive activities of 11beta- hydroxysteroid dehydrogenase in rat Leydig cells. *Endocrinology* 138 156-161.

Garcea G, Lloyd TD, Gescher A, Dennison AR, Steward WP & Berry DP 2004 Angiogenesis of gastrointestinal tumours and their metastases - a target for intervention? *European Journal of Cancer* 40 1302-1313.

Gardiner P, Schrode K, Quinlan D, Martin BK, Boreham DR, Rogers MS, Stubbs K, Smith M & Karim A 1989 Spironolactone metabolism: steady-state serum levels of the sulfur-containing metabolites. *Journal of Clinical Pharmacology* 29 342-347.

Gaynon PS & Lustig RH 1995 The use of glucocorticoids in acute lymphoblastic leukemia of childhood. Molecular, cellular, and clinical considerations. *Journal of Pediatratric Hematology and Oncology.* 17 1-12.

Gazit Y, Baish JW, Safabakhsh N, Leunig M, Baxter LT & Jain RK 1997 Fractal characteristics of tumor vascular architecture during tumor growth and regression. *Microcirculation.* 4 395-402.

Gille J, Reisinger K, Westphal-Varghese B & Kaufmann R 2001 Decreased mRNA stability as a mechanism of glucocorticoid-mediated inhibition of vascular endothelial growth factor gene expression by cultured keratinocytes. *Journal of Investigative Dermatology.* 117 1581-1587.

Glasper ER & DeVries AC 2005 Social structure influences effects of pair-housing on wound healing. *Brain Behaviour and Immunity.* 19 61-68.

Gohel AR, Hand AR & Gronowicz GA 1995 Immunogold localization of beta 1-integrin in bone: effect of glucocorticoids and insulin-like growth factor I on integrins and osteocyte formation. *Journal of Histochemistry and Cytochemistry.* 43 1085-1096.

Gordon CB, Li DG, Stagg CA, Manson P & Udelsman R 1994 Impaired wound healing in Cushing's syndrome: the role of heat shock proteins. *Surgery* 116 1082-1087.

Graiani G, Emanueli C, Desortes E, Van Linthout S, Pinna A, Figueroa CD, Manni L & Madeddu P 2004 Nerve growth factor promotes reparative angiogenesis and inhibits endothelial apoptosis in cutaneous wounds of Type 1 diabetic mice. *Diabetologia* 47 1047-1054.

Gronowicz GA & McCarthy MB 1995 Glucocorticoids inhibit the attachment of osteoblasts to bone extracellular matrix proteins and decrease beta 1-integrin levels. *Endocrinology* 136 598-608.

Grose R, Werner S, Kessler D, Tuckermann J, Huggel K, Durka S, Reichardt HM & Werner S 2002 A role for endogenous glucocorticoids in wound repair. *EMBO Reports.* 3 575-582.

Gruss CJ, Satyamoorthy K, Berking C, Lininger J, Nesbit M, Schaider H, Liu ZJ, Oka M, Hsu MY, Shirakawa T, Li G, Bogenrieder T, Carmeliet P, El Deiry WS, Eck SL, Rao JS, Baker AH, Bennet JT, Crombleholme TM, Velazquez O, Karmacharya J, Margolis DJ, Wilson JM, Detmar M, Skobe M, Robbins PD, Buck C & Herlyn M 2003 Stroma formation and angiogenesis by overexpression of growth factors, cytokines, and proteolytic enzymes in human skin grafted to SCID mice. *Journal of Investigative Dermatology.* 120 683-692.

Grynkiewicz G, Poenie M & Tsien RY 1985b A new generation of Ca2+ indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* 260 3440-3450.

Gu YC, Talts JF, Gullberg D, Timpl R & Ekblom M 2001 Glucocorticoids down-regulate the extracellular matrix proteins fibronectin, fibulin-1 and fibulin-2 in bone marrow stroma. *European Journal of Haematology.* 67 176-184.

Guggino S, Mechine NA, Weltin D, Imbs JL & Stephan D 2003 [Anti-angiogenic effects of aldosterone antagonists in the fibrin chamber in rats]. *Archives des maladies du cœur et des vaisseaux..* 96 808-810.

Guggino S, Weltin D, Chapelon D, Imbs JL & Stephan D 2002 [Anti-angiogenesis effects of aldosterone antagonist diuretics]. *Archives des Maladies du Cœur et des Vaisseaux.* 95 727-731.

Haas TL, Milkiewicz M, Davis SJ, Zhou AL, Egginton S, Brown MD, Madri JA & Hudlicka O 2000 Matrix metalloproteinase activity is required for activity-induced angiogenesis in rat skeletal muscle. *American Journal of Physiology - Heart and Circulatory Physiology* 279 H1540-H1547.

Hadoke PWF, Christy C, Kotelevtsev YV, Williams BC, Kenyon CJ, Seckl JR, Mullins JJ & Walker BR 2001 Endothelial cell dysfunction in mice after transgenic knockout of type 2, but not type 1, 11β-hydroxysteroid dehydrogenase. *Circulation* 104 2832-2837.

Hafezi-Moghadam A, Simoncini T, Yang E, Limbourg FP, Plumier JC, Rebsamen MC, Hsieh CM, Chui DS, Thomas KL, Prorock AJ, Laubach VE, Moskowitz MA, French BA, Ley K & Liao JK 2002 Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nature Medicine.* 8 473-479.

Hague S, MacKenzie IZ, Bicknell R & Rees MC 2002 In-vivo angiogenesis and progestogens. *Human Reproduction.* 17 786-793.

Hammami MM & Siiteri PK 1991 Regulation of 11β-hydroxysteroid dehydrogenase activity in human skin fibroblasts: enzymatic modulation of glucocorticoid action. *Journal of Clinical Endocrinology and Metabolism* 73 326-334.

Hammond GL, Smith CL, Paterson NAM & Sibbald WJ 1990 A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. *Journal of Clinical Endocrinology and Metabolism* 71 34-39.

Hanemaaijer R, Koolwijk P, le Clercq L, de Vree WJ & van Hinsbergh VW 1993 Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells. Effects of tumour necrosis factor alpha, interleukin 1 and phorbol ester. *Biochemical Journal* 296 (Pt 3) 803-809.

Hao X, Mansson-Broberg A, Blomberg P, Dellgren G, Siddiqui AJ, Grinnemo KH, Wardell E & Sylven C 2004 Angiogenic and cardiac functional effects of dual gene transfer of VEGF-A165 and PDGF-BB after myocardial infarction. *Biochemical and Biophysical Research Communications* 322 292-296.

Harris HJ, Kotelevtsev YV, Mullins JJ, Seckl JR & Holmes MC 2001 11β-Hydroxysteroid dehydrogenase type 1 null mice have altered hypothalamic-pituitary-adrenal axis activity: a novel control of glucocorticoid feedback. *Endocrinology* 142 114-120.

Hasan Q, Tan ST, Gush J, Peters SG & Davis PF 2000 Steroid therapy of a proliferating hemangioma: histochemical and molecular changes. *Pediatrics* 105 117-120.

Hasan Q, Tan ST, Xu B & Davis PF 2003 Effects of five commonly used glucocorticoids on haemangioma in vitro. *Clinical and Experimental Pharmacology and Physiology* 30 140-144.

Hatakeyama H, Inaba S & Miyamori I 1999 11beta-Hydroxysteroid dehydrogenase in cultured human vascular cells: Possible role in the development of hypertension. *Hypertension* 33 1179-1184.

Heba G, Krzeminski T, Porc M, Grzyb J, Ratajska A & Dembinska-Kiec A 2001 The time course of tumor necrosis factor-alpha, inducible nitric oxide synthase and vascular endothelial growth factor expression in an experimental model of chronic myocardial infarction in rats. *Journal of Vascular Research.* 38 288-300.

Heikinheimo O, Pesonen U, Huupponen R, Koulu M & Lahteenmaki P 1994 Hepatic metabolism and distribution of mifepristone and its metabolites in rats. *Human Reproduction.* 9 Suppl 1 40-46.

Heiniger CD, Kostadinova RM, Rochat MK, Serra A, Ferrari P, Dick B, Frey BM & Frey FJ 2003 Hypoxia causes down-regulation of 11 beta-hydroxysteroid dehydrogenase type 2 by induction of Egr-1. *Federation of American Societies for Experimental Biology Journal* 17 917-919.

Heiniger CD, Rochat MK, Frey FJ & Frey BM 2001 TNF-alpha enhances intracellular glucocorticoid availability. *FEBS Letters.* 507 351-356.

Helisch A & Schaper W 2003 Arteriogenesis: the development and growth of collateral arteries. *Microcirculation.* 10 83-97.

Hernandez MV, Guanabens N, Alvarez L, Monegal A, Peris P, Riba J, Ercilla G, Martinez de Osaba MJ & Munoz-Gomez J 2004 Immunocytochemical evidence on the effects of glucocorticoids on type I collagen synthesis in human osteoblastic cells. *Calcified Tissue Internation.* 74 284-293.

Herschman HR 1994 Regulation of prostaglandin synthase-1 and prostaglandin synthase-2. *Cancer Metastasis Reviews.* 13 241-256.

Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, Dyspersin GD, Cleutjens JP, Shipley M, Angellilo A, Levi M, Nube O, Baker A, Keshet E, Lupu F, Herbert JM, Smits JF, Shapiro SD, Baes M, Borgers M, Collen D, Daemen MJ & Carmeliet P 1999 Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nature Medicine.* 5 1135-1142.

Hillier SG & Tetsuka M 1998 An anti-inflammatory role for glucocorticoids in the ovaries? *Journal of Reproductive Immunology* 39 21-27.

Hirschi KK & D'Amore PA 1996 Pericytes in the microvasculature. *Cardiovascular Research* 32 687-698.

Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP & Jain RK 1998 Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. *Proceedings of the National Academy of Sciences U.S.A* 95 4607-4612.

Hodgson DM, Behfar A, Zingman LV, Kane GC, Perez-Terzic C, Alekseev AE, Puceat M & Terzic A 2004 Stable benefit of embryonic stem cell therapy in myocardial infarction. *American Journal Physiology- Heart and Circulatory Physiology* 287 H471-H479.

Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Coller BS, Teitelbaum S & Hynes RO 1999 Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *Journal of Clinical Investigation* 103 229-238.

Hoefnagels WHL & Kloppenborg PWC 1983 Antimineralocorticoid effects of dexamethasone in subjects treated with glycyrrhetinic acid. *Journal of Hypertension* 1 (Suppl 2) 313-315.

Hori Y, Hu DE, Yasui K, Smither RL, Gresham GA & Fan TP 1996 Differential effects of angiostatic steroids and dexamethasone on angiogenesis and cytokine levels in rat sponge implants. *British Journal of Pharmacology* 118 1584-1591.

Huang J & Kontos CD 2002 PTEN modulates vascular endothelial growth factor-mediated signaling and angiogenic effects. *Journal of Biological Chemistry* 277 10760-10766.

Huminiecki L, Gorn M, Suchting S, Poulsom R & Bicknell R 2002 Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. *Genomics* 79 547-552.

Huwer H, Rissland J, Vollmar B, Nikoloudakis N, Welter C, Menger MD & Schafers HJ 1999 Angiogenesis and microvascularization after cryothermia-induced myocardial infarction: a quantitative fluorescence microscopic study in rats. *Basic Research in Cardiology.* 94 85-93.

Hynes RO 2002 A reevaluation of integrins as regulators of angiogenesis. *Nature Medicine.* 8 918-921.

Illanes J, Dabancens A, Acuna O, Fuenzalida M, Guerrero A, Lopez C & Lemus D 2002 Effects of betamethasone, sulindac and quinacrine drugs on the inflammatory neoangiogenesis response induced by polyurethane sponge implanted in mouse. *Biological.Research.* 35 339-345.

Ingber DE, Madri JA & Folkman J 1986 A possible mechanism for inhibition of angiogenesis by angiostatic steroids: induction of capillary basement membrane dissolution. *Endocrinology* 119 1768-1775.

Ismail JA, Poppa V, Kemper LE, Scatena M, Giachelli CM, Coffin JD & Murry CE 2003 Immunohistologic labeling of murine endothelium. *Cardiovascular Pathology.* 12 82-90.

Iurlaro M, Scatena M, Zhu WH, Fogel E, Wieting SL & Nicosia RF 2003 Rat aorta-derived mural precursor cells express the Tie2 receptor and respond directly to stimulation by angiopoietins. *Journal of Cellular Science.* 116 3635-3643.

Iwakura A, Fujita M, Kataoka K, Tambara K, Sakakibara Y, Komeda M & Tabata Y 2003 Intramyocardial sustained delivery of basic fibroblast growth factor improves angiogenesis and ventricular function in a rat infarct model. *Heart and Vessels* 18 93-99.

Jacobson L 2005 Hypothalamic-pituitary-adrenocortical axis regulation. *Endocrinology and Metabolism Clinics of North America.* 34 271-92, vii.

Jacobson L & Sapolsky R 1991 The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocrine Reviews* 12 118-134.

Jacoby RC, Owings JT, Ortega T, Gosselin R & Feldman EC 2001 Biochemical basis for the hypercoagulable state seen in Cushing syndrome; discussion 1006-7. *Archives in Surgery.* 136 1003-1006.

Jain RK 2003 Molecular regulation of vessel maturation. *Nature Medicine.* 9 685-693.

Jamieson PM, Chapman KE, Edwards CRW & Seckl JR 1995 11β-Hydroxysteroid dehydrogenase is an exclusive 11β-reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. *Endocrinology* 136 4754-4761.

Jamieson PM, Chapman KE & Seckl JR 1999 Tissue- and temporal-specific regulation of 11beta-hydroxysteroid dehydrogenase type 1 by glucocorticoids in vivo. *Journal of Steroid Biochemistry and Molecular Biology* 68 245-250.

Jamieson PM, Walker BR, Chapman KE, Rossiter S & Seckl JR 2000 11β-Hydroxysteroid dehydrogenase type 1 is a predominant 11-reductase in the intact perfused rat liver. *Journal of Endocrinology* 175 685-692.

Jiang L, Jha V, Dhanabal M, Sukhatme VP & Alper SL 2001 Intracellular Ca(2+) signaling in endothelial cells by the angiogenesis inhibitors endostatin and angiostatin. *American Journal of Physiology Cell Physiology* 280 C1140-C1150.

Johns DG, Dorrance AM, Tramontini NL & Webb RC 2001 Glucocorticoids inhibit tetrahydrobiopterin-dependent endothelial function. *Experimental Biology and Medicine* 226 27-31.

Johnson PJ, Ganjam VK, Slight SH, Kreeger JM & Messer NT 2004 Tissue-specific dysregulation of cortisol metabolism in equine laminitis. *Equine Veterinary Journal.* 36 41-45.

Jones MK, Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ & Tarnawski AS 1999 Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. *Nature Medicine.* 5 1418-1423.

Kalluri R 2002 Discovery of type IV collagen non-collagenous domains as novel integrin ligands and endogenous inhibitors of angiogenesis. *Cold Spring Harbor Symposia on Quantitative Biology.* 67 255-266.

Kataoka S, Kudo A, Hirano H, Kawakami H, Kawano T, Higashihara E, Tanaka H, Delarue F, Sraer JD, Mune T, Krozowski ZS & Yan K 2002 11beta-hydroxysteroid dehydrogenase type 2 is expressed in the human kidney glomerulus. *Journal of Clinical Endocrinology and Metabolism* 87 877-882.

Kawabata H, Takada K & Katori R 1995 Effect of methylprednisolone on metabolism and contractility in the stunned myocardium. *Angiology* 46 895-904.

Kayisli UA, Mahutte NG & Arici A 2002 Uterine chemokines in reproductive physiology and pathology. *American Journal of Reproductive Immunology and microbiology.* 47 213-221.

Kenouch S, Lombes M, Delahaye F, Eugene E, Bonvalet J-P & Farman N 1994 Human skin as target for aldosterone: Coexpression of mineralocorticoid receptors and 11beta-hydroxysteroid dehydrogenas. *Journal of Clinical Endocrinology and Metabolism* 79 1334-1341.

Kenyon BM, Voest EE, Chen CC, Flynn E, Folkman J & D'Amato RJ 1996 A model of angiogenesis in the mouse cornea. *Invest Ophthalmology and Visual Science.* 37 1625-1632.

Kerstens MN, Navis G & Dullaart RP 2003 Does a reduced 11 beta HSD type 2 activity contribute to sodium retention in the nephrotic syndrome? *Nephrology Dialysis Transplantation.* 18 620-621.

King KL & Cidlowski JA 1998 Cell cycle regulation and apoptosis. *Annual Reviews in Physiology* 60 601-617.

Klauber N, Browne F, Anand-Apte B & D'Amato RJ 1996 New activity of spironolactone. Inhibition of angiogenesis in vitro and in vivo. *Circulation* 94 2566-2571.

Klekamp JG, Jarzecka K, Hoover RL, Summar ML, Redmond N & Perkett EA 1997 Vascular endothelial growth factor is expressed in ovine pulmonary vascular smooth muscle cells in vitro and regulated by hypoxia and dexamethasone. *Pediatric Research* 42 744-749.

Knedler A & Ham RG 1987 Optimized medium for clonal growth of human microvascular endothelial cells with minimal serum. *In Vitro Cellular and Developmental Biology.* 23 481-491.

Kodama T, Shimizu N, Yoshikawa N, Makino Y, Ouchida R, Okamoto K, Hisada T, Nakamura H, Morimoto C & Tanaka H 2003 Role of the glucocorticoid receptor for regulation of hypoxia-dependent gene expression. *Journal of Biological Chemistry* 278 33384-33391.

Kohli A, Leech M, Vain P, Laurie DA & Christou P 1998 Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. *Proceedings of the National Academy of Sciences.U.S.A* 95 7203-7208.

Kolonin M, Pasqualini R & Arap W 2001 Molecular addresses in blood vessels as targets for therapy. *Current Opinion in Chemical Biology.* 5 308-313.

Kones RJ 1975 Glucocorticoid therapy for acute myocardial infarction. *Acta Cardiologica.* 30 353-374.

Kornel L, Kanamarlapudi N, Travers T, Taff DJ, Patel N, Chen C, Baum RM & Raynor WJ 1982 Studies on high affinity binding of mineralo- and glucocorticoids in rabbit aorta cytosol. *Journal of Steroid Biochemistry* 16 245-264.

Kotelevtsev YV, Brown RW, Fleming S, Edwards CRW, Seckl JR & Mullins JJ 1999 Hypertension in mice caused by inactivation of 11β-hydroxysteroid dehydrogenase type 2. *Journal of Clinical Investigation* 103 683-689.

Kotelevtsev YV, Holmes MC, Burchell A, Houston PM, Scholl D, Jamieson PM, Best R, Brown RW, Edwards CRW, Seckl JR & Mullins JJ 1997 11β-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid inducible responses and resist hyperglycaemia on obesity and stress. *Proceedings of the National Academy of Sciences USA* 94 14924-14929.

Kraling BM, Wiederschain DG, Boehm T, Rehn M, Mulliken JB & Moses MA 1999 The role of matrix metalloproteinase activity in the maturation of human capillary endothelial cells in vitro. *Journal of Cell Sciences.* 112 (Pt 10) 1599-1609.

Krozowski Z, Stuchberry S, White PC, Monder C & Funder JW 1990 Characterisation of 11β-hydroxysteroid dehydrogenase gene expression: identification of multiple unique forms of messenger ribonucleic acid in the rat kidney. *Endocrinology* 127 3009-3013.

Kucharz EJ 1988 Hormonal control of collagen metabolism--Part I. *Endocrinologie* 26 69-79.

Kyossev Z, Walker PD & Reeves WB 1996 Immunolocalization of NAD-dependent 11beta-hydroxysteroid dehydrogenase in human kidney and colon. *Kidney International* 49 271-281.

Lan NC, Karin M, Nguyen T, Weisz A, Birnbaum MJ, Eberhardt NL & Baxter JD 1984 Mechanisms of glucocorticoid hormone action. *Journal of Steroid Biochemistry* 20 77-88.

Lanz B, Kadereit B, Ernst S, Shojaati K, Causevic M, Frey BM, Frey FJ & Mohaupt MG 2003 Angiotensin II regulates 11beta-hydroxysteroid dehydrogenase type 2 via AT2 receptors. *Kidney International* 64 970-977.

Lanz C-B, Causevic M, Heiniger C, Frey FJ, Frey BM & Mohaupt MG 2001 Fluid shear stress reduces 11beta-hydroxysteroid dehydrogenase type 2. *Hypertension* 37 160-169.

Laue L, Kawai S, Brandon DD, Brightwell D, Barnes K, Knazek RA, Loriaux DL & Chrousos GP 1988 Receptor-mediated effects of glucocorticoids on inflammation: enhancement of the inflammatory response with a glucocorticoid antagonist. *Journal of Steroid Biochemistry* 29 591-598.

Laue L, Lotze MT, Chrousos GP, Barnes K, Loriaux DL & Fleisher TA 1990 Effect of chronic treatment with the glucocorticoid antagonist RU 486 in man: toxicity, immunological, and hormonal aspects. *Journal of Clinical Endocrinology and Metabolism* 71 1474-1480.

Le Menuet D, Viengchareun S, Muffat-Joly M, Zennaro MC & Lombes M 2004 Expression and function of the human mineralocorticoid receptor: lessons from transgenic mouse models. *Molecular and Cellular Endocrinology.* 217 127-136.

Leahy KM, Koki AT & Masferrer JL 2000 Role of cyclooxygenases in angiogenesis. *Current Medicinal Chemistry.* 7 1163-1170.

Leckie CM, Welberg LM & Seckl JR 1998 11beta-hydroxysteroid dehydrogenase is a predominant reductase in intact rat leydig cells. *Journal of Endocrinology* 159 233-238.

Lee PC, Salyapongse AN, Bragdon GA, Shears LL, Watkins SC, Edington HD & Billiar TR 1999 Impaired wound healing and angiogenesis in eNOS-deficient mice. *American Journal of Physiology* 277 H1600-H1608.

Leech M, Huang XR, Morand EF & Holdsworth SR 2000a Endogenous glucocorticoids modulate experimental anti-glomerular basement membrane glomerulonephritis. *Clinical and Experimental Immunology.* 119 161-168.

Leech M, Metz C, Bucala R & Morand EF 2000b Regulation of macrophage migration inhibitory factor by endogenous glucocorticoids in rat adjuvant-induced arthritis. *Arthritis Rheumatism.* 43 827-833.

LeGal YM & Morrissey LL 1990 Methylprednisolone interventions in myocardial infarction: a controversial subject. *Canadian Journal of Cardiology* 6 405-410.

Lemus D, Dabancens A, Illanes J, Fuenzalida M, Guerrero A & Lopez C 2001 Antiangiogenic effect of betamethasone on the chick cam stimulated by TA3 tumor supernatant. *Biological Research.* 34 227-236.

Leyssac PP, Christensen P, Hill R & Skinner SL 1975 Indomethacin blockade of renal PGE-synthesis: effect on total renal and tubular function and plasma renin concentration in hydropenic rats and on their response to isotonic saline. *Acta Physiologica Scandinavica.* 94 484-496.

Li J, Zhang YP & Kirsner RS 2003 Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix. *Microscopy Research and Technique.* 60 107-114.

Li Q, Li B, Wang X, Leri A, Jana KP, Liu Y, Kajstura J, Baserga R & Anversa P 1997 Overexpression of insulin-like growth factor-1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy. *Journal of Clinical Investigation* 100 1991-1999.

Liekens S, De Clercq E & Neyts J 2001 Angiogenesis: regulators and clinical applications. *Biochemical Pharmacology* 61 253-270.

Lincoff AM, Furst JG, Ellis SG, Tuch RJ & Topol EJ 1997 Sustained local delivery of dexamethasone by a novel intravascular eluting stent to prevent restenosis in the porcine coronary injury model. *Journal of the American College of Cardiology* 29 808-816.

Lindahl P, Hellstrom M, Kalen M & Betsholtz C 1998 Endothelial-perivascular cell signaling in vascular development: lessons from knockout mice. *Current Opinion in Lipidology.* 9 407-411.

Liposits Z & Bohn MC 1993 Association of glucocorticoid receptor immunoreactivity with cell membrane and transport vesicles in hippocampal and hypothalamic neurons of the rat. *Journal of Neuroscience Research.* 35 14-19.

Liu Y, Nakagawa Y, Wang Y, Li R, Li X, Ohzeki T & Friedman TC 2003 Leptin activation of corticosterone production in hepatocytes may contribute to the reversal of obesity and hyperglycemia in leptin-deficient ob/ob mice. *Diabetes* 52 1409-1416.

Liu Y-J, Nakagawa Y, Nasuda K, Saegusa H & Igarashi Y 1996 Effect of growth hormone, insulin and dexamethasone on 11beta-hydroxysteroid dehydrogenase activity on a primary culture of rat hepatocytes. *Life Sciences* 59 227-234.

Livingstone DEW, Jones GC, Smith K, Andrew R, Kenyon CJ & Walker BR 2000a Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. *Endocrinology* 141 560-563.

Livingstone DEW, Kenyon CJ & Walker BR 2000b Mechanisms of dysregulation of 11β-hydroxysteroid dehydrogenase type 1 in Zucker obese rats. *Journal of Endocrinology* 167 533-539.

Lloyd-MacGilp SA, Nelson SM, Florin M, Lo M, McKinnell J, Sassard J & Kenyon CJ 1999 11β-Hydroxysteroid dehydrogenase and corticosteroid action in Lyon hypertensive rats. *Hypertension* 34 1123-1128.

Longenecker JP, Kilty LA & Johnson LK 1982 Glucocorticoid influence on growth of vascular wall cells in culture. *Journal of Cellular Physiology* 113 197-202.

Longenecker JP, Kilty LA & Johnson LK 1984 Glucocorticoid inhibition of vascular smooth muscle cell proliferation: influence of homologous extracellular matrix and serum mitogens. *Journal of Cell Biology.* 98 534-540.

Lopez JJ, Laham RJ, Stamler A, Pearlman JD, Bunting S, Kaplan A, Carrozza JP, Sellke FW & Simons M 1998 VEGF administration in chronic myocardial ischemia in pigs. *Cardiovascular Research* 40 272-281.

Low SC, Chapman KE, Edwards CRW, Wells T, Robinson ICAF & Seckl JR 1994a Sexual dimorphism of hepatic 11β-hydroxysteroid dehydrogenase in the rat: the role of growth hormone patterns. *Journal of Endocrinology* 143 541-548.

Low SC, Moisan M-P, Edwards CRW & Seckl JR 1994b Glucocorticoids regulate 11β-hydroxysteroid dehydrogenase activity and gene expression in vivo in the rat. *Journal of Neuroendocrinology* 6 285-290.

Lu L, Zhang JQ, Ramires FJ & Sun Y 2004 Molecular and cellular events at the site of myocardial infarction: from the perspective of rebuilding myocardial tissue. *Biochemical and Biophysical Research Communications* 320 907-913.

Lutgens E, Daemen MJ, de Muinck ED, Debets J, Leenders P & Smits JF 1999 Chronic myocardial infarction in the mouse: cardiac structural and functional changes. *Cardiovascular Research* 41 586-593.

MacKenzie MA, Hoefnagels WHL, Jansen RWMM, Benraad TJ & Kloppenborg PWC 1990 The influence of glycyrrhetinic acid on plasma cortisol and cortisone in healthy young volunteers. *Journal of Clinical Endocrinology and Metabolism* 70 1637-1643.

Malmberg K 1997 Prospective randomised study of intensive insulin treatment on long term survival after acute myocardial infarction in patients with diabetes mellitus. DIGAMI (Diabetes Mellitus, Insulin Glucose Infusion in Acute Myocardial Infarction) Study Group. *British Medical Journal* 314 1512-1515.

Mao H, Yin KS, Wang ZL, Li FY, Zhang XL, Liu CT & Lei S 2004 Effects of glucocorticoid and cysteinyl leukotriene 1 receptor antagonist on CD(34+) hematopoietic cells in bone marrow of asthmatic mice. *Chinese Medical Journal (English.)* 117 592-597.

Maragoudakis ME, Sarmonika M & Panoutsacopoulou M 1989 Antiangiogenic action of heparin plus cortisone is associated with decreased collagenous protein

synthesis in the chick chorioallantoic membrane system. *Journal of Pharmacology and Experimental Therapeutics* 251 679-682.

Marinos RS, Zhang W, Wu G, Kelly KA & Meininger CJ 2001 Tetrahydrobiopterin levels regulate endothelial cell proliferation. *Am.J.Physiol Heart Circ.Physiol* 281 H482-H489.

Masferrer JL & Seibert K 1994 Regulation of prostaglandin synthesis by glucocorticoids. *Receptor* 4 25-30.

Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR & Flier JS 2001 A transgenic model of visceral obesity and the metabolic syndrome. *Science* 294 2166-2170.

McEwen BS, de Kloet ER & Rostene W 1986 Adrenal steroid receptors and actions in the nervous system. *Physiological Reviews* 66 1121-1188.

McLaughlin AP & De Vries GW 2001 Role of PLCgamma and Ca(2+) in. *American Journal of Physiology Cell Physiology* 281 C1448-C1456.

McNatt LG, Weimer L, Yanni J & Clark AF 1999 Angiostatic activity of steroids in the chick embryo CAM and rabbit cornea models of neovascularization. *Journal of Ocular Pharmacology and Therapeutics.* 15 413-423.

Michael LH, Ballantyne CM, Zachariah JP, Gould KE, Pocius JS, Taffet GE, Hartley CJ, Pham TT, Daniel SL, Funk E & Entman ML 1999 Myocardial infarction and remodeling in mice: effect of reperfusion. *American Journal of Physiology* 277 H660-H668.

Michalopoulos GK, Bowen WC, Mule K & Luo J 2003 HGF-, EGF-, and dexamethasone-induced gene expression patterns during formation of tissue in hepatic organoid cultures. *Gene Expression.* 11 55-75.

Moisan M-P, Edwards CRW & Seckl JR 1992a Differential promoter usage by the rat 11β-hydroxysteroid dehydrogenase gene. *Molecular Endocrinology* 6 1082-1087.

Moisan M-P, Edwards CRW & Seckl JR 1992b Ontogeny of 11β-hydroxysteroid dehydrogenase in rat brain and kidney. *Endocrinology* 130 400-404.

Moldow RL, Beck KD, Weaver S & Servatius RJ 2005 Blockage of glucocorticoid, but not mineralocorticoid receptors prevents the persistent increase in circulating basal corticosterone concentrations following stress in the rat. *Neuroscience Letters.* 374 25-28.

Monder C & Lakshmi V 1989 Evidence for kinetically distinct forms of corticosteroid 11β-dehydrogenase in rat liver microsomes. *Journal of Steroid Biochemistry* 32 77-83.

Montrella-Waybill M, Clore JN, Schoolwerth AC & Watlington CO 1991 Evidence that high dose cortisol-induced Na+ retention in man is not mediated by the

mineralocorticoid receptor. *Journal of Clinical Endocrinology and Metabolism* 72 1060-1066.

Mora A, Davies AM, Bertrand L, Sharif I, Budas GR, Jovanovic S, Mouton V, Kahn CR, Lucocq JM, Gray GA, Jovanovic A & Alessi DR 2003 Deficiency of PDK1 in cardiac muscle results in heart failure and increased sensitivity to hypoxia. *EMBO Journal.* 22 4666-4676.

Morton NM, Holmes MC, Fievet C, Staels B, Tailleux A, Mullins JJ & Seckl JR 2001 Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11β-hydroxysteroid dehydrogenase type 1 null mice. *Journal of Biological Chemistry* 276 41293-41300.

Moskowitz RM, Burns JJ, DiCarlo EF, Flaim SF, Harrison TS, Peuler J & Zelis R 1979 Cage size and exercise affects infarct size in rat after coronary artery cauterization. *Journal of Applied Physiology* 47 393-396.

Nagashima M, Asano G & Yoshino S 2000 Imbalance in production between vascular endothelial growth factor and endostatin in patients with rheumatoid arthritis. *Journal of Rheumatology.* 27 2339-2342.

Napolitano A, Voice MW, Edwards CW, Seckl JR & Chapman KE 1998 11beta-Hydroxysteroid dehydrogenase 1 in adipocytes: Expression is differentiation-dependent and hormonally regulated. *Journal of Steroid Biochemistry and Molecular Biology* 64 251-260.

Nauck M, Karakiulakis G, Perruchoud AP, Papakonstantinou E & Roth M 1998 Corticosteroids inhibit the expression of the vascular endothelial growth factor gene in human vascular smooth muscle cells. *European Journal of Pharmacology* 341 309-315.

Newman PJ & Newman DK 2003 Signal transduction pathways mediated by PECAM-1: new roles for an old molecule in platelet and vascular cell biology. *Arteriosclerosis Thrombosis and Vascular Biology.* 23 953-964.

Nicosia RF, Belser P, Bonanno E & Diven J 1991 Regulation of angiogenesis in vitro by collagen metabolism. *In Vitro Cellular and Developmental Biology.* 27A 961-966.

Nicosia RF, Bonanno E & Villaschi S 1992 Large-vessel endothelium switches to a microvascular phenotype during angiogenesis in collagen gel culture of rat aorta. *Atherosclerosis* 95 191-199.

Nicosia RF, Lin YJ, Hazelton D & Qian X 1997 Endogenous regulation of angiogenesis in the rat aorta model. Role of vascular endothelial growth factor. *Amercian Journal of Pathology.* 151 1379-1386.

Nicosia RF, Nicosia SV & Smith M 1994a Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro. *Amercian Journal of Pathology.* 145 1023-1029.

Nicosia RF & Ottinetti A 1990 Growth of microvessels in serum-free matrix culture of rat aorta. A quantitative assay of angiogenesis in vitro. *Laboratory Investigation* 63 115-122.

Nicosia RF, Villaschi S & Smith M 1994b Isolation and characterization of vasoformative endothelial cells from the rat aorta. *In Vitro Cellular and Developmental Biology-Animal* 30A 394-399.

Nilius B & Droogmans G 2001 Ion channels and their functional role in vascular endothelium. *Physiology Reviews.* 81 1415-1459.

Nissanov J, Tuman RW, Gruver LM & Fortunato JM 1995 Automatic vessel segmentation and quantification of the rat aortic ring assay of angiogenesis. *Laboratory Investigation* 73 734-739.

Nwe KH, Morat PB & Khalid BK 1997 Opposite effects of sex steroids on 11beta-hydroxysteroid dehydrogenase activity in the normal and adrenalectomized rat testis. *General Pharmacology* 28 661-664.

Nyirenda MJ, Lindsay RS, Kenyon CJ, Burchell A & Seckl JR 1998 Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. *Journal of Clinical Investigation* 101 2174-2181.

Oakley RH, Sar M & Cidlowski JA 1996 The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. *Journal of Biological Chemistry* 271 9550-9559.

Oberfield SE, Levine LS, Carey RM, Greig F, Ulick S & New MI 1983 Metabolic and blood pressure responses to hydrocortisone in the syndrome of apparent mineralocorticoid excess. *Journal of Clinical Endocrinology and Metabolism* 56 332-339.

Oishi Y, Fu ZW, Ohnuki Y, Kato H & Noguchi T 2002 Molecular basis of the alteration in skin collagen metabolism in response to in vivo dexamethasone treatment: effects on the synthesis of collagen type I and III, collagenase, and tissue inhibitors of metalloproteinases. *British Journal of Dermatology* 147 859-868.

Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A & Anversa P 2001 Bone marrow cells regenerate infarcted myocardium. *Nature* 410 701-705.

Ortega N & Werb Z 2002 New functional roles for non-collagenous domains of basement membrane collagens. *Journal of Cell Science.* 115 4201-4214.

Padgett DA, Marucha PT & Sheridan JF 1998 Restraint stress slows cutaneous wound healing in mice. *Brain Behaviour and Immunity.* 12 64-73.

Painson JC, Thorner MO, Krieg RJ & Tannenbaum GS 1992 Short-term adult exposure to estradiol feminizes the male pattern of spontaneous and growth

hormone-releasing factor-stimulated growth hormone secretion in the rat. *Endocrinology* 130 511-519.

Papapetropoulos A, Desai KM, Rudic RD, Mayer B, Zhang R, Ruiz-Torres MP, Garcia-Cardena G, Madri JA & Sessa WC 1997 Nitric oxide synthase inhibitors attenuate transforming-growth-factor-beta 1-stimulated capillary organization in vitro. *American Journal of Pathology.* 150 1835-1844.

Parks LL, Turney MK, Gaitan D & Kovacs WJ 1998 Expression of 11beta-hydroxysteroid dehydrogenase type 2 in an ACTH-producing small cell lung cancer. *Journal of Steroid Biochemistry and Molecular Biology* 67 341-346.

Paterson JM, Morton NM, Fievet C, Kenyon CJ, Holmes MC, Staels B, Seckl JR & Mullins JJ 2004 Metabolic syndrome without obesity: Hepatic overexpression of 11beta-hydroxysteroid dehydrogenase type 1 in transgenic mice. *Proceedings of the National Academy of Sciences U.S.A* 101 7088-7093.

Patrassi GM, Dal Bo ZR, Boscaro M, Martinelli S & Girolami A 1985 Further studies on the hypercoagulable state of patients with Cushing's syndrome. *Thrombosis and Haemostasis.* 54 518-520.

Patrassi GM, Sartori MT, Livi U, Casonato A, Danesin C, Vettore S & Girolami A 1997 Impairment of fibrinolytic potential in long-term steroid treatment after heart transplantation. *Transplantation* 64 1610-1614.

Penhaligon M & Camplejohn RS 1985 Combination heparin plus cortisone treatment of two transplanted tumors in C3H/He mice. *Journal of North Atlantic Cancer Institute.* 74 869-873.

Pepper MS 2001 Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscleosis and Thrombosis and Vascular Biology.* 21 1104-1117.

Perretti M & Ahluwalia A 2000 The microcirculation and inflammation: site of action for glucocorticoids. *Microcirculation.* 7 147-161.

Pfeffer MA & Braunwald E 1990 Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation* 81 1161-1172.

Pitt B, White H, Nicolau J, Martinez F, Gheorghiade M, Aschermann M, van Veldhuisen DJ, Zannad F, Krum H, Mukherjee R & Vincent J 2005 Eplerenone reduces mortality 30 days after randomization following acute myocardial infarction in patients with left ventricular systolic dysfunction and heart failure. *Journal of the American College of Cardiology* 46 425-431.

Pitzalis C, Pipitone N & Perretti M 2002 Regulation of leukocyte-endothelial interactions by glucocorticoids. *Annals New York Academy of Sciences* 966 108-118.

Plant TD & Schaefer M 2003 TRPC4 and TRPC5: receptor-operated Ca2+-permeable nonselective cation channels. *Cell Calcium* 33 441-450.

Pocock TM, Foster RR & Bates DO 2004 Evidence of a role for TRPC channels in VEGF-mediated increased vascular permeability in vivo. *American Journal of Physiology -Heart Circulatory Physiology* 286 H1015-H1026.

Polat A, Nayci A, Polat G & Aksoyek S 2002 Dexamethasone down-regulates endothelial expression of intercellular adhesion molecule and impairs the healing of bowel anastomoses. *European Journal of Surgery.* 168 500-506.

Pratt, W. B. The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. Journal of Biological Chemistry 268, 21455-21458. 1993.

Preissner KT, May AE, Wohn KD, Germer M & Kanse SM 1997 Molecular crosstalk between adhesion receptors and proteolytic cascades in vascular remodelling. *Thrombosis and Haemostasis.* 78 88-95.

Profita M, Gagliardo R, Di Giorgi R, Bruno A, Riccobono L, Bonanno A, Bousquet J & Vignola AM 2004 In vitro effects of flunisolide on MMP-9, TIMP-1, fibronectin, TGF-beta1 release and apoptosis in sputum cells freshly isolated from mild to moderate asthmatics. *Allergy* 59 927-932.

Pross C, Farooq MM, Lane JS, Angle N, Tomono CK, Xavier AE, Freischlag JA, Collins AE, Law RE & Gelabert HA 2002 Rat and human aortic smooth muscle cells display differing migration and matrix metalloproteinase activities in response to dexamethasone. *Journal of Vascular Surgery.* 35 1253-1259.

Pugh CW & Ratcliffe PJ 2003 Regulation of angiogenesis by hypoxia: role of the HIF system. *Nature Medicine.* 9 677-684.

Puolakkainen PA, Bradshaw AD, Brekken RA, Reed MJ, Kyriakides T, Funk SE, Gooden MD, Vernon RB, Wight TN, Bornstein P & Sage EH 2005 SPARC-thrombospondin-2-double-null mice exhibit enhanced cutaneous wound healing and increased fibrovascular invasion of subcutaneous polyvinyl alcohol sponges. *Journal of Histochemistry and Cytochemistry.* 53 571-581.

Rabbitt EH, Gittoes NJ, Stewart PM & Hewison M 2003 11beta-hydroxysteroid dehydrogenases, cell proliferation and malignancy. *Journal of Steroid Biochemistry and Molecular Biology* 85 415-421.

Radke PW, Weber C, Kaiser A, Schober A & Hoffmann R 2004 Dexamethasone and restenosis after coronary stent implantation: new indication for an old drug? *Current Pharmaceutical Design* 10 349-355.

Radomski MW, Palmer RMJ & Moncada S 1990 Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proceedings of the National Academy of Sciences USA* 87 10043-10047.

Rae MT, Niven D, Ross A, Forster T, Lathe R, Critchley HO, Ghazal P & Hillier SG 2004 Steroid signalling in human ovarian surface epithelial cells: the response to interleukin-1alpha determined by microarray analysis. *Journal of Endocrinology* 183 19-28.

Raff H 1987 Glucocorticoid inhibition of neurohypophysial vasopressin secretion. *American Journal of Physiology* 252 R635-R644.

Rafii S, Meeus S, Dias S, Hattori K, Heissig B, Shmelkov S, Rafii D & Lyden D 2002 Contribution of marrow-derived progenitors to vascular and cardiac regeneration. *Seminars in Cell Developmental Biology.* 13 61-67.

Rajan V, Edwards CR & Seckl JR 1996 11 beta-Hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. *Journal of Neuroscience* 16 65-70.

Ramanujan S, Koenig GC, Padera TP, Stoll BR & Jain RK 2000 Local imbalance of proangiogenic and antiangiogenic factors: a potential mechanism of focal necrosis and dormancy in tumors. *Cancer Research* 60 1442-1448.

Rask E, Walker BR, Soderberg S, Livingstone DE, Eliasson M, Johnson O, Andrew R & Olsson T 2002 Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11beta-hydroxysteroid dehydrogenase type 1 activity. *Journal of Clinical Endocrinology and Metabolism* 87 3330-3336.

Rees DD, Cellek S, Palmer RMJ & Moncada S 1990 Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxic shock. *Biochemical and Biophysical Research Communications* 173 541-547.

Reimers B, Moussa I, Akiyama T, Kobayashi Y, Albiero R, Di Francesco L, Di Mario C & Colombo A 1998 Persistent high restenosis after local intrawall delivery of long-acting steroids before coronary stent implantation. *Journal of Invasive Cardiology* 10 323-331.

Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, Sheppard D, Hynes RO & Hodivala-Dilke KM 2002 Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. *Nature Medicine.* 8 27-34.

Ricketts ML, Shoesmith KJ, Hewison M, Strain A, Eggo MC & Stewart PM 1998 Regulation of 11beta-hydroxysteroid dehydrogenase type 1 in primary cultures of rat and human hepatocytes. *Journal of Endocrinology* 156 159-168.

Rohan RM, Fernandez A, Udagawa T, Yuan J & D'Amato RJ 2000 Genetic heterogeneity of angiogenesis in mice. *Federation of American Societies for Experimental Biology Journal* 14 871-876.

Roman CD, Choy H, Nanney L, Riordan C, Parman K, Johnson D & Beauchamp RD 2002 Vascular endothelial growth factor-mediated angiogenesis inhibition and postoperative wound healing in rats. *Journal of Surgical Research.* 105 43-47.

Ryu JS, Majeska RJ, Ma Y, LaChapelle L & Guller S 1999 Steroid regulation of human placental integrins: suppression of alpha2 integrin expression in cytotrophoblasts by glucocorticoids. *Endocrinology* 140 3904-3908.

Sakai RR, Lakshmi V, Monder C & McEwen BS 1992 Immunocytochemical localization of 11beta-hydroxysteroid dehydrogenase in hippocampus and other brain regions of the rat. *Journal of Neuroendocrinology* 4 101-106.

Sakamoto N, Tanaka NG, Tohgo A, Osada Y & Ogawa H 1987 Inhibitory effects of heparin plus cortisone acetate on endothelial cell growth both in cultures and in tumor masses. *Journal of North Atlantic Cancer Institute.* 78 581-585.

Sandeep TC & Walker BR 2001 Pathophysiology of modulation of local glucocorticoid levels by 11beta-hydroxysteroid dehydrogenases. *Trends in Endocrinology and Metabolism* 12 446-453.

Sapan CV, Lundblad RL & Price NC 1999 Colorimetric protein assay techniques. *Biotechnology and Applied Biochemistry.* 29 (Pt 2) 99-108.

Saruta T, Suzuki H, Handa M, Igarashi Y, Kondo K & Senba S 1986 Multiple factors contribute to the pathogenesis of hypertension in Cushing's syndrome. *Journal of Clinical Endocrinology and Metabolism* 62 275-279.

Sasano H, Ohashi Y, Suzuki T & Nagura H 1998 Vascularity in human adrenal cortex. *Modern Pathology.* 11 329-333.

Scheuer DA & Mifflin SW 1997 Chronic corticosterone treatment increases myocardial infarct size in rats with ischemia-reperfusion injury. *American Journal of Physiology* 272 R2017-R2024.

Schoeler D, Grutzkau A, Henz BM, Kuchler J & Kruger-Krasagakis S 2003 Interleukin-6 enhances whereas tumor necrosis factor alpha and interferons inhibit integrin expression and adhesion of human mast cells to extracellular matrix proteins. *Journal of Investigative Dermatology.* 120 795-801.

Schulz M, Eggert M, Baniahmad A, Dostert A, Heinzel T & Renkawitz R 2002 RU486-induced glucocorticoid receptor agonism is controlled by the receptor N terminus and by corepressor binding. *Journal of Biological Chemistry* 277 26238-26243.

Schwarz ER, Speakman MT, Patterson M, Hale SS, Isner JM, Kedes LH & Kloner RA 2000 Evaluation of the effects of intramyocardial injection of DNA expressing vascular endothelial growth factor (VEGF) in a myocardial infarction model in the rat--angiogenesis and angioma formation. *Journal of the American College of Cardiology* 35 1323-1330.

Seckl JR & Walker BR 2001 11β-Hydroxysteroid dehydrogenase type 1 - a tissue-specific amplifier of glucocorticoid action. *Endocrinology* 142 1371-1376.

Semenza GL 2003 Angiogenesis in ischemic and neoplastic disorders. *Annual Reviews in Medicine.* 54 17-28.

Shackleton CHL, Honour JW, Dillon MJ, Chantler C & Jones RWA 1980 Hypertension in a four-year-old child; gas chromatographic and mass spectrophotometric evidence for deficient hepatic metabolism of steroids. *Journal of Clinical Endocrinology and Metabolism* 50 786-792.

Sheppard KE 2003 Corticosteroid receptors, 11 beta-hydroxysteroid dehydrogenase, and the heart. *Vitamins and Hormones.* 66 77-112.

Sheppard KE & Autelitano DJ 2002 11Beta-hydroxysteroid dehydrogenase 1 transforms 11-dehydrocorticosterone into transcriptionally active glucocorticoid in neonatal rat heart. *Endocrinology* 143 198-204.

Sheppard KE, Khoo K, Krozowski ZS & Li KZ 1998 Steroid specificity of the putative DHB receptor: Evidence that the receptor is not 11betaHSD. *American Journal of Physiology - Endocrinology & Metabolism* 275 E124-E131.

Shields PP, Dixon JE & Glembotski CC 1988 The secretion of atrial natriuretic factor-(99-126) by cultured cardiac myocytes is regulated by glucocorticoids. *Journal of Biological Chemistry* 263 12619-12628.

Simmons WW, Ungureanu-Longrois D, Smith GK, Smith TW & Kelly RA 1996 Glucocorticoids regulate inducible nitric oxide synthase by inhibiting tetrahydrobiopterin synthesis and L-Arginine transport. *Journal of Biological Chemistry* 271 23928-23937.

Singer AJ & Clark RA 1999 Cutaneous wound healing. *New England Journal of Medicine* 341 738-746.

Slight S, Ganjam VK, Nonneman DJ & Weber KT 1993 Glucocorticoid metabolism in the cardiac interstitium: 11 beta-hydroxysteroid dehydrogenase activity in cardiac fibroblasts. *Journal of Laboratory and Clinical Medicine.* 122 180-187.

Smith RE & Funder JW 1991 Renal 11β-hydroxysteroid dehydrogenase activity: effects of age, sex and altered hormonal status. *Journal of Steroid Biochemistry and Molecular Biology* 38 265-267.

Smith RE, Maguire JA, Stein-Oakley AN, Sasano H, Takahashi K, Fukushima K & Krozowski ZS 1996 Localization of 11beta-hydroxysteroid dehydrogenase type II in human epithelial tissues. *Journal of Clinical Endocrinology and Metabolism* 81 3244-3248.

Soff GA, Sanderowitz J, Gately S, Verrusio E, Weiss I, Brem S & Kwaan HC 1995 Expression of plasminogen activator inhibitor type 1 by human prostate carcinoma cells inhibits primary tumor growth, tumor-associated angiogenesis, and metastasis to lung and liver in an athymic mouse model. *Journal of Clinical Investigation* 96 2593-2600.

Solomon AJ & Gersh BJ 1993 The results and indications of acute revascularization in myocardial infarction. *Coronary Artery Disease.* 4 1068-1075.

Sottile J 2004 Regulation of angiogenesis by extracellular matrix. *Biochimica Biophysica Acta* 1654 13-22.

Souness GW, Brem AS & Morris DJ 2002 11 beta-hydroxysteroid dehydrogenase antisense affects vascular contractile response and glucocorticoid metabolism. *Steroids* 67 195-201.

Staton CA, Stribbling SM, Tazzyman S, Hughes R, Brown NJ & Lewis CE 2004 Current methods for assaying angiogenesis in vitro and in vivo. *Int.J.Exp.Pathol.* 85 233-248.

Stewart PM, Corrie JET, Shackleton CHL & Edwards CRW 1988 Syndrome of apparent mineralocorticoid excess: a defect in the cortisol-cortisone shuttle. *Journal of Clinical Investigation* 82 340-349.

Stewart PM, Krozowski ZS, Gupta A, Milford DV, Howie AJ, Sheppard MC & Whorwood CB 1996 Hypertension in the syndrome of apparent mineralocorticoid excess due to mutation of the 11β-hydroxysteroid dehydrogenase type 2 gene. *Lancet* 347 88-91.

Stewart PM, Murry BA & Mason JI 1994 Human kidney 11β-hydroxysteroid dehydrogenase is a high affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type 1 isoform. *Journal of Clinical Endocrinology and Metabolism* 79 480-484.

Stewart PM, Valentino R, Wallace AM, Burt D, Shackleton CHL & Edwards CRW 1987 Mineralocorticoid activity of liquorice: 11β-hydroxysteroid dehydrogenase deficiency comes of age. *Lancet* ii 821-824.

Stewart PM, Whorwood CB & Mason JI 1995 Type 2 11beta-hydroxysteroid dehydrogenase in foetal and adult life. *Journal of Steroid Biochemistry and Molecular Biology* 55 465-471.

Strecker EP, Gabelmann A, Boos I, Lucas C, Xu Z, Haberstroh J, Freudenberg N, Stricker H, Langer M & Betz E 1998 Effect on intimal hyperplasia of dexamethasone released from coated metal stents compared with non-coated stents in canine femoral arteries. *Cardiovascular and Interventional Radiology* 21 487-496.

Streif JU, Nahrendorf M, Hiller KH, Waller C, Wiesmann F, Rommel E, Haase A & Bauer WR 2005 In vivo assessment of absolute perfusion and intracapillary blood volume in the murine myocardium by spin labeling magnetic resonance imaging. *Magnectic Resonance in Medicine.* 53 584-592.

Sullivan DC & Bicknell R 2003 New molecular pathways in angiogenesis. *British Journal of Cancer* 89 228-231.

Symes JF, Losordo DW, Vale PR, Lathi KG, Esakof DD, Mayskiy M & Isner JM 1999 Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. *Annals of Thoracic Surgery.* 68 830-836.

Takahashi K-I, Sasano H, Fukushima K, Hirasawa G, Miura H, Sasaki I, Matsuno S, Krozowski ZS & Nagura H 1998 11 beta-hydroxysteroid dehydrogenase type II in human colon: A new marker of fetal development and differentiation in neoplasms. *Anticancer Research* 18 3381-3388.

Takeda Y, Miyamori I, Yoneda T, Iki K, Hatakeyama H & Takeda R 1994a Gene expression of 11β-hydroxysteroid dehydrogenase in the mesenteric arteries of genetically hypertensive rats. *Hypertension* 23 577-580.

Takeda Y, Miyamori I, Yoneda T, Ito Y & Takeda R 1994b Expression of 11β-hydroxysteroid dehydrogenase mRNA in rat vascular smooth muscle cells. *Life Sciences* 54 281-285.

Tcacencu I 2002 Mifepristone (RU-486) impairs post-surgical wound healing of the larynx. *Medicine Science Monitor.* 8 BR397-BR400.

Teelucksingh S, Mackie ADR, Burt D, McIntyre MA, Brett L & Edwards CRW 1990 Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *Lancet* 335 1060-1063.

Tetsuka M, Haines LC, Milne M, Simpson GE & Hillier SG 1999 Regulation of 11beta-hydroxysteroid dehydrogenase type 1 gene expression by LH and interleukin-1beta in cultured rat granulosa cells. *Journal of Endocrinology* 163 417-423.

Thieringer R, Le Grand CB, Carbin L, Cai TQ, Wong B, Wright SD & Hermanowski-Vosatka A 2001 11 Beta-hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages. *Journal of Immunology.* 167 30-35.

Thomas FJ, Thomas MJ, Tetsuka M, Mason JI & Hillier SG 1998 Corticosteroid metabolism in human granulosa-lutein cells. *Clinical Endocrinology (Oxford)* 48 509-513.

Thompson WD, Harvey JA, Kazmi MA & Stout AJ 1991 Fibrinolysis and angiogenesis in wound healing. *Journal of Pathology.* 165 311-318.

Tjandra K, Kubes P, Rioux K & Swain MG 1996 Endogenous glucocorticoids inhibit neutrophil recruitment to inflammatory sites in cholestatic rats. *American Journal of Physiology* 270 G821-G825.

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C & Brown RA 2002 Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nature Reviews in Molecular and Cell Biology.* 3 349-363.

Tomlinson JW, Moore J, Cooper MS, Bujalska I, Shahmanesh M, Burt C, Strain A, Hewison M & Stewart PM 2001 Regulation of expression of 11β-hydroxysteroid

dehydrogenase type 1 in adipose tissue: tissue-specific induction by cytokines. *Endocrinology* 142 1982-1989.

Tomlinson JW, Moore JS, Clark PM, Holder G, Shakespeare L & Stewart PM 2004a Weight loss increases 11beta-hydroxysteroid dehydrogenase type 1 expression in human adipose tissue. *Journal of Clinical Endocrinology and Metabolism* 89 2711-2716.

Tomlinson JW, Walker EA, Bujalska IJ, Draper N, Lavery GG, Cooper MS, Hewison M & Stewart PM 2004b 11beta-hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. *Endocrine Reviews* 25 831-866.

Topper JN & Gimbrone MA, Jr. 1999 Blood flow and vascular gene expression: fluid shear stress as a modulator of endothelial phenotype. *Molecular Medicine Today.* 5 40-46

Trepel M, Arap W & Pasqualini R 2002 In vivo phage display and vascular heterogeneity: implications for targeted medicine. *Curr.Opin.Chem.Biol.* 6 399-404.

Ulick S, Levine LS, Gunczler P, Zanconato G, Ramirez LC, Rauh W, Rosler A, Bradlow HL & New MI 1979 A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *Journal of Clinical Endocrinology and Metabolism* 49 757-764.

Ullian ME 1999 The role of corticosteroids in the regulation of vascular tone. *Cardiovascular Research* 41 55-64.

Ullian ME, Hazen-Martin DJ, Walsh LG, Davda RK & Egan BM 1996 Carbenoxolone damages endothelium and enhances vasoconstrictor action in aortic rings. *Hypertension* 27 1346-1352.

Valen G, Kawakami T, Tahepold P, Starkopf J, Kairane C, Dumitrescu A, Lowbeer C, Zilmer M & Vaage J 2000 Pretreatment with methylprednisolone protects the isolated rat heart against ischaemic and oxidative damage. *Free Radical Research.* 33 31-43.

van Royen N, Hoefer I, Bottinger M, Hua J, Grundmann S, Voskuil M, Bode C, Schaper W, Buschmann I & Piek JJ 2003 Local monocyte chemoattractant protein-1 therapy increases collateral artery formation in apolipoprotein E-deficient mice but induces systemic monocytic CD11b expression, neointimal formation, and plaque progression. *Circulation Research* 92 218-225.

Vassalle C, Domenici C, Lubrano V & L'Abbate A 2003 Interaction between nitric oxide and cyclooxygenase pathways in endothelial cells. *Journal of Vascular Research.* 40 491-499.

Versaci F, Gaspardone A, Tomai F, Ribichini F, Russo P, Proietti I, Ghini AS, Ferrero V, Chiariello L, Gioffre PA, Romeo F & Crea F 2002 Immunosuppressive Therapy for the Prevention of Restenosis after Coronary Artery Stent Implantation (IMPRESS Study). *Journal of the American College of Cardiology* 40 1935-1942.

Villa AE, Guzman LA, Chen W, Golomb G, Levy RJ & Topol EJ 1994 Local delivery of dexamethasone for prevention of neointimal proliferation in a rat model of balloon angioplasty. *Journal of Clinical Investigation* 93 1243-1249.

Villaschi S & Nicosia RF 1993 Angiogenic role of endogenous basic fibroblast growth factor released by rat aorta after injury. *American Journal of Pathology.* 143 181-190.

Villaschi S, Nicosia RF & Smith MR 1994 Isolation of a morphologically and functionally distinct smooth muscle cell type from the intimal aspect of the normal rat aorta. Evidence for smooth muscle cell heterogeneity. *In Vitro Cell Developmental Biology -Animal* 30A 589-595.

Vinson RB, Carroll JL & Pruett SB 1998 Mechanism of suppressed neutrophil mobilization in a mouse model for binge drinking: role of glucocorticoids. *American Journal of Physiology* 275 R1049-R1057.

Virag JI & Murry CE 2003 Myofibroblast and endothelial cell proliferation during murine myocardial infarct repair. *American Journal of Pathology.* 163 2433-2440.

Voyta JC, Via DP, Butterfield CE & Zetter BR 1984 Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *Journal of Cell Biology.* 99 2034-2040.

Vyden JK, Nagasawa K, Rabinowitz B, Parmley WW, Tomoda H, Corday E & Swan HJ 1974 Effects of methylprednisolone administration in acute myocardial infarction. *American Journal of Cardiology* 34 677-686.

Wade CE, Spitz IM, Lahteenmaki P, Heikinheimo O, Krieger DT & Bardin CW 1988 Effects of the antiglucocorticoid RU 486 on adrenal function in dogs. *Journal of Clinical Endocrinology and Metabolism* 66 473-479.

Wake DJ, Rask E, Livingstone DE, Soderberg S, Olsson T & Walker BR 2003 Local and systemic impact of transcriptional up-regulation of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue in human obesity. *Journal of Clinical Endocrinology and Metabolism* 88 3983-3988.

Wake DJ & Walker BR 2004 11 beta-hydroxysteroid dehydrogenase type 1 in obesity and the metabolic syndrome. *Molecular and Cellular Endocrinology.* 215 45-54.

Walker BR, Connacher AA, Lindsay RM, Webb DJ & Edwards CRW 1995 Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation. *Journal of Clinical Endocrinology and Metabolism* 80 3155-3159.

Walker BR, Sang KS, Williams BC & Edwards CRW 1994a Direct and indirect effects of carbenoxolone on responses to glucocorticoids and noradrenaline in rat aorta. *Journal of Hypertension* 12 33-39.

Walker BR & Williams BC 1992 Corticosteroids and vascular tone: mapping the messenger maze. *Clinical Science* 82 597-605.

Walker BR, Williams BC & Edwards CRW 1994b Regulation of 11β-hydroxysteroid dehydrogenase activity by the hypothalamic-pituitary-adrenal axis in the rat. *Journal of Endocrinology* 141 467-472.

Walker BR, Yau JL, Brett LP, Seckl JR, Monder C, Williams BC & Edwards CRW 1991 11β-Hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids. *Endocrinology* 129 3305-3312.

Walker EA, Clark AM, Hewison M, Ride JP & Stewart PM 2001 Functional expression, characterization, and purification of the catalytic domain of human 11-beta -hydroxysteroid dehydrogenase type 1. *Journal of Biological Chemistry* 276 21343-21350.

Wallerath T, Witte K, Schafer SC, Schwartz PM, Prelwitz W, Wohlfart P, Kleinert H, Lehr HA, Lemmer B & Forstermann U 1999 Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension. *Proceedings of the National Academy of Sciences USA* 92 13357-13362.

Walsh DA 1999 Angiogenesis and arthritis. *Rheumatology.(Oxford)* 38 103-112.

Wang D, Liu YH, Yang XP, Rhaleb NE, Xu J, Peterson E, Rudolph AE & Carretero OA 2004 Role of a selective aldosterone blocker in mice with chronic heart failure. *Journal of Cardiac Failure* 10 67-73.

Wang YS, Friedrichs U, Eichler W, Hoffmann S & Wiedemann P 2002 Inhibitory effects of triamcinolone acetonide on bFGF-induced migration and tube formation in choroidal microvascular endothelial cells. *Graefes Archive for Clinical and Experimental Ophthalmology.* 240 42-48.

Wen FQ, Liu X, Manda W, Terasaki Y, Kobayashi T, Abe S, Fang Q, Ertl R, Manouilova L & Rennard SI 2003 TH2 Cytokine-enhanced and TGF-beta-enhanced vascular endothelial growth factor production by cultured human airway smooth muscle cells is attenuated by IFN-gamma and corticosteroids. *Journal of Allergy and Clinical Immunology.* 111 1307-1318.

Wexler BC 1976 Adrenocortical suppression and myocardial infarction in non-arteriosclerotic (virgin) and arteriosclerotic (breeder) rats. *Atherosclerosis* 23 393-411.

Whitworth JA 1994 Studies on the mechanism of glucocorticoid hypertension in humans. *Blood Pressure* 3 24-32.

Whitworth JA, Schyvens CG, Zhang Y, Andrews MC, Mangos GJ & Kelly JJ 2002 The nitric oxide system in glucocorticoid-induced hypertension. *Journal of Hypertension* 20 1035-1043.

Whorwood CB, Sheppard MC & Stewart PM 1993 Tissue specific effects of thyroid hormone on 11β-hydroxysteroid dehydrogenase gene expression. *Journal of Steroid Biochemistry and Molecular Biology* 46 539-547.

Wielckens K, Delfs T, Muth A, Freese V & Kleeberg HJ 1987 Glucocorticoid-induced lymphoma cell death: the good and the evil. *Journal of Steroid Biochemistry* 27 413-419.

Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY & Jain RK 2004 Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nature Medicine.* 10 145-147.

Yamamoto T, Terada N, Nishizawa Y & Petrow V 1994 Angiostatic activities of medroxyprogesterone acetate and its analogues. *International Journal of Cancer* 56 393-399.

Yamamoto Y, Ishizu A, Ikeda H, Otsuka N & Yoshiki T 2004 Dexamethasone increased plasminogen activator inhibitor-1 expression on human umbilical vein endothelial cells: an additive effect to tumor necrosis factor-alpha. *Pathobiology* 71 295-301.

Yang F, Liu YH, Yang XP, Xu J, Kapke A & Carretero OA 2002 Myocardial infarction and cardiac remodelling in mice. *Experimental Physiology* 87 547-555.

Yang K, Smith CL, Dales D, Hammond GL & Challis JR 1992 Cloning of an ovine 11beta-hydroxysteroid dehydrogenase complementary deoxyribonucleic acid: tissue and temporal distribution of its messenger ribonucleic acid during fetal and neonatal development. *Endocrinology* 131 2120-2126.

Yang S & Zhang L 2004 Glucocorticoids and vascular reactivity. *Current Vascular Pharmacology.* 2 1-12.

Yau JLW, Noble JM, Kenyon CJ, Hibberd C, Kotelevtsev YV, Mullins JJ & Seckl JR 2001 Lack of tissue glucocorticoid reactivation in 11ß-hydroxysteroid dehydrogenase type 1 knockout mice ameliorates age-related learning impairments. *Proceedings of the National Academy of Sciences USA* 98 4716-4721.

Yossuck P, Yan Y, Tadesse M & Higgins RD 2001 Dexamethasone alters TNF-alpha expression in retinopathy. *Molecular Genetics and Metabolism* 72 164-167.

Ytrehus K & Downey JM 1993 Experimental models assessing the physiology of myocardial ischaemia. *Current Opinion in Cardiology* 8 581.

Zawicki DF, Jain RK, Schmid-Schoenbein GW & Chien S 1981 Dynamics of neovascularization in normal tissue. *Microvascular Research* 21 27-47.

Zeller W, Meier G, Burki K & Panoussis B 1998 Adverse effects of tribromoethanol as used in the production of transgenic mice. *Laboratory Animals* 32 407-413.

Zhang GQ, Ye ZH, Shi YE, He CH, Bai XM & Xu JQ 1991 [HPLC of RU 486 and its metabolites in human blood]. *Shengzhi Yu Biyun.* 11 26-31.

Zhang Z, Tarone G & Turner DC 1993 Expression of integrin alpha 1 beta 1 is regulated by nerve growth factor and dexamethasone in PC12 cells. Functional consequences for adhesion and neurite outgrowth. *Journal of Biological Chemistry* 268 5557-5565.

Zhao X, Lu X & Feng Q 2002 Deficiency in endothelial nitric oxide synthase impairs myocardial angiogenesis. *American Journal of Physiology- Heart and Circulatory Physiology* 283 H2371-H2378.

Ziche M, Ruggiero M, Pasquali F & Chiarugi VP 1985 Effects of cortisone with and without heparin on angiogenesis induced by prostaglandin E1 and by S180 cells, and on growth of murine transplantable tumours. *International. Journal of Cancer* 35 549-552.

Zoellner H, Wojta J, Gallicchio M, McGrath K & Hamilton JA 1993 Cytokine regulation of the synthesis of plasminogen activator inhibitor-2 by human vascular endothelial cells. Comparison with plasminogen activator inhibitor-1 synthesis. *Thrombosis and Haemostasis.* 69 135-140.

Zoppi N, Ghinelli A, Gardella R, Barlati S & Colombi M 1998 Effect of dexamethasone on the assembly of the matrix of fibronectin and on its receptors organization in Ehlers-Danlos syndrome skin fibroblasts. *Cell Biology International.* 22 499-508.

Preventing local regeneration of glucocorticoids by 11β-hydroxysteroid dehydrogenase type 1 enhances angiogenesis

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Edited by Judah Folkman, Harvard Medical School, Boston, MA, and approved June 30, 2005 (received for review January 25, 2005)

Angiogenesis restores blood flow to healing tissues, a process that is inhibited by high doses of glucocorticoids. However, the role of endogenous glucocorticoids and the potential for antiglucocorticoid therapy to enhance angiogenesis is unknown. Using *in vitro* and *in vivo* models of angiogenesis in mice, we examined effects of (*i*) endogenous glucocorticoids, (*ii*) blocking endogenous glucocorticoid action with the glucocorticoid receptor antagonist RU38486, and (*iii*) abolishing local regeneration of glucocorticoids by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1). Glucocorticoids, administered at physiological concentrations, inhibited angiogenesis in an *in vitro* aortic ring model and *in vivo* in polyurethane sponges implanted s.c. RU38486-enhanced angiogenesis in s.c. sponges, in healing surgical wounds, and in the myocardium of mice 7 days after myocardial infarction induced by coronary artery ligation. 11βHSD1 knockout mice showed enhanced angiogenesis *in vitro* and *in vivo* within sponges, wounds, and infarcted myocardium. Endogenous glucocorticoids, including those generated locally by 11βHSD1, exert tonic inhibition of angiogenesis. Inhibition of 11βHSD1 in liver and adipose has been advocated to reduce cardiovascular risk in the metabolic syndrome: these data suggest that 11βHSD1 inhibition offers a previously uncharacterized therapeutic approach to improve healing of ischemic or injured tissue.

myocardial infarction | wound healing

Angiogenesis, the formation of new vessels from existing ones, is a key factor in many common diseases (1–4), and manipulation of angiogenesis is an important therapeutic target (5, 6). Supraphysiological concentrations of glucocorticoids have been used *in vitro* and *in vivo* to inhibit angiogenesis (7–11). It is unknown, however, whether physiological concentrations of endogenous glucocorticoids (principally cortisol in humans and corticosterone in mice) regulate angiogenesis.

The influence of glucocorticoids on their target tissues is regulated in a tissue-specific manner by the isozymes of 11β-hydroxysteroid dehydrogenase (11βHSD) (12). 11βHSD type 1 functions predominantly as an 11-oxidoreductase converting inactive 11-keto metabolites (cortisone in humans; 11-dehydrocorticosterone in mice) into active 11-hydroxy glucocorticoids (cortisol and corticosterone) (13). 11βHSD-1 is highly expressed in liver, adipose tissue, and regions of the central nervous system, where it amplifies intracellular glucocorticoid concentrations and thereby maintains glucocorticoid receptor activation (13). 11βHSD type 2 is an exclusive 11β-dehydrogenase, inactivating cortisol or corticosterone in distal nephron, colon, and sweat glands, thus preventing inappropriate access of glucocorticoids to mineralocorticoid receptors (12). Both 11βHSD isozymes are expressed in the blood vessel wall (14–18). In mouse and rat aorta, 11βHSD-2 is localized in endothelial cells and 11βHSD-1 in vascular smooth muscle (15, 16).

Glucocorticoids have diverse effects on vascular function, altering vasoconstrictor responses (19), impairing endothelium-dependent vasodilatation (19), and inhibiting inflammation and cell proliferation (20, 21). We recently reported studies of vascular function in knockout mice deficient in either 11βHSD isozyme (22). In aortae from 11βHSD-2 −/− mice, endothelium-dependent vasodilatation was impaired, suggesting that 11βHSD-2 protects endothelial cell receptors from glucocorticoids. However, there was no abnormality of vascular tone in 11βHSD-1 −/− mice, so the role of the type 1 isozyme in the vessel wall remained unclear. At that time, Cai *et al.* (23) demonstrated that 11βHSD1 expression in vascular smooth muscle is up-regulated in response to proinflammatory cytokines, raising the possibility that increased local generation of glucocorticoids contributes to feedback regulation of vascular inflammation.

Given that inflammatory cytokines can promote angiogenesis (24) and pharmacological doses of glucocorticoids have antiangiogenic activity, we hypothesized that 11βHSD-1 modulates angiogenesis by determining the local regeneration of active glucocorticoid in the vessel wall. If so, then manipulation of 11βHSD-1 may provide a novel therapeutic tool to alter angiogenesis. Here, we have tested this hypothesis by using *in vitro*, *in vivo*, and pathological models of angiogenesis in mice.

Methods

Mice. Male, C57Bl6J wild-type and 11βHSD-1 homozygous null (−/−) mice aged 8–10 weeks were used (Charles River Laboratories). Genetic inactivation of 11βHSD-1 has been described in MF-1/129 mice (25); for the current experiments, mice were backcrossed over >10 generations onto a C57Bl6J background (26).

Aortic Ring Preparations. Mice were killed, and thoracic aortae were removed, washed in serum-free MCDB 131 medium (Invitrogen), cleaned of periadventitial tissue, and divided into 1- to 3-mm rings.

11βHSD activities were measured by incubating wild-type aortic rings for 24 h at 37°C in 1 ml of DMEM-F12 medium (Invitrogen) containing ^{3}H-steroid supplemented with FBS (1%), streptomycin (100 μg/ml), penicillin (100 units/ml), and amphotericin (0.25 μg/ml) (27). 11β-Reductase activity was determined by adding 10 pmol [$^3H_4$]-11-dehydrocorticosterone [synthesized in-house from 1,2,6,7-[$^3H_4$]-corticosterone (Amersham Pharmacia Biosciences) by using rat placental homogenate]. Mouse liver (28 ± 5 mg) and medium alone were used as positive and negative controls, respectively. 11β-Dehydrogenase activity was determined by adding 10 pmol 1,2,6,7-[$^3H_4$]-corticosterone. Mouse kidney (13 ± 3 mg) and medium alone served as positive and negative controls. After incubation,

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1.

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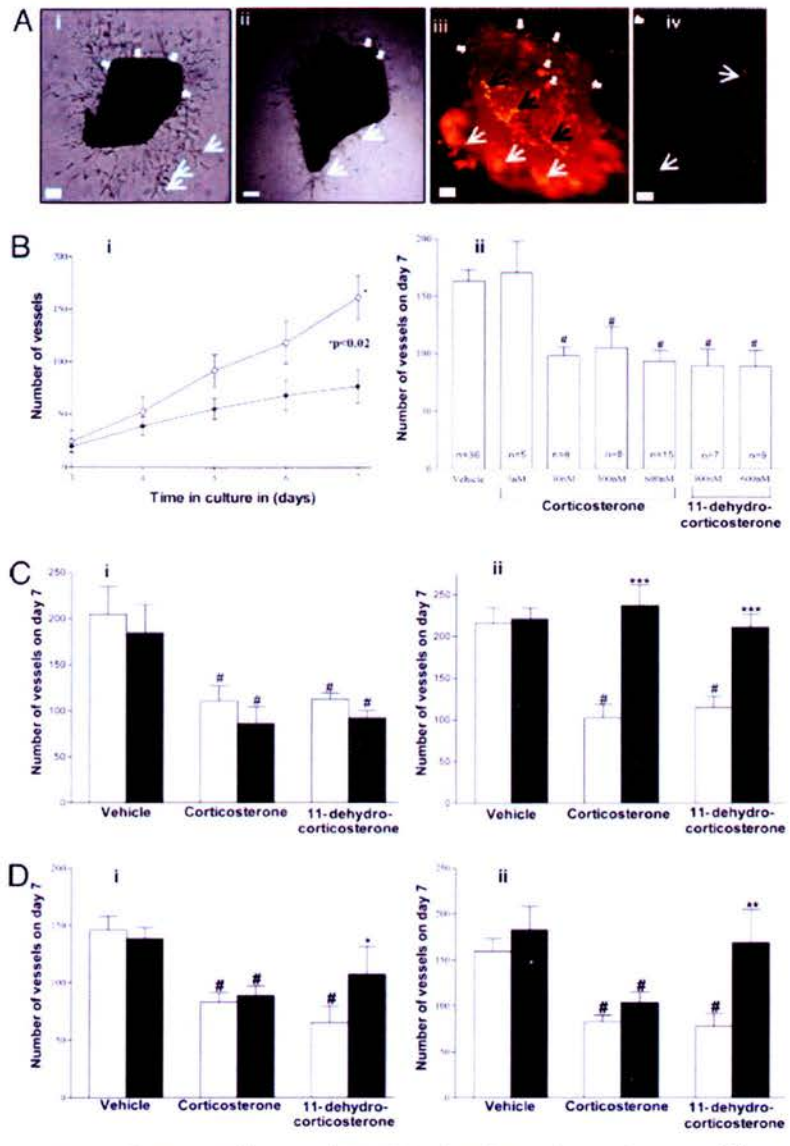

Fig. 1. Angiogenesis in aortic rings *in vitro*. (*A*) Light microscopy of new vessels shown sprouting from aortic rings. (*i*) Aortic ring incubated for 7 days without glucocorticoid. (*ii*) Aortic ring incubated for 7 days in the presence of glucocorticoid. Thick white arrows indicate the aortic ring; thin white arrows indicate new vessels. (Scale bar: 0.2 mm.) (*iii*) Uptake of low-density lipoprotein (LDL) is shown by fluorescence microscopy. This ring was incubated for 7 days without steroids. Thick white arrows indicate the aortic ring; thin white arrows indicate uptake of fluorescent labeled LDL in endothelial cells in new vessels; black arrows indicate uptake in endothelial cells of the aortic ring. (Scale bar: 0.2 mm.) (*iv*) High power view of new vessels; thick white arrows indicate the aortic ring and thin white arrows indicate uptake of fluorescent labeled low density lipoprotein in endothelial cells (Scale bar: 0.02 mm.) (*Bi*) Time course and effect of corticosterone on angiogenesis. ○, results from vessels incubated without steroids; ◆, results from vessels incubated with corticosterone (600 nM). Results are mean ± SEM for $n = 4$ per group. Comparison was by repeated measures ANOVA; *, $P < 0.02$. (*Bii*) Effects of corticosterone and 11-dehydrocorticosterone. Vessels were counted after 7-day incubation with steroids. Results are mean ± SEM. #, $P < 0.01$ versus vehicle by 2-way ANOVA and least squares difference post hoc test. (*C*) Influence of receptor antagonism. (*i*) Effects of the mineralocorticoid receptor antagonist spironolactone. Aortic rings from C57Bl6J mice were incubated with (filled bars) and without (open bars) spironolactone (10^{-6} M) and glucocorticoids (600 nM). Results are mean ± SEM for $n = 6$ experiments. #, $P < 0.02$ versus corresponding vehicle. Spironolactone alone had no effect. (*ii*) Effects of the glucocorticoid receptor antagonist RU38486. Aortic rings from C57Bl6 mice were incubated with (filled bars) and without (open bars) RU38486 (10^{-6} M) and glucocorticoids (600 nM). Results are mean ± SEM for $n = 4$–6 experiments. # $P < 0.01$ versus corresponding vehicle. ***, $P < 0.001$ for the effect of RU38486 in the presence of glucocorticoid. RU38486 alone had no effect. (*D*) Effects of 11βHSD inhibition (*i*) Pharmacological inhibitor carbenoxolone. Aortic rings from C57Bl6J mice were incubated with (filled bars) and without (open bars) carbenoxolone (10^{-6} M) and glucocorticoids (600 nM). Results are mean ± SEM for $n = 5$ experiments. #, $P < 0.01$ versus corresponding vehicle. *, $P < 0.04$ for the effect of carbenoxolone in the presence of 11-dehydrocorticosterone. Carbenoxolone had no effect in the presence of corticosterone or vehicle alone. (*ii*) Transgenic deletion of 11βHSD1. Effects of corticosterone and 11-dehydrocorticosterone on angiogenesis in vessels from 11βHSD1 −/− mice. Aortic rings from C57Bl6J wild-type (open bars) or 11βHSD1 −/− (filled bars) mice were incubated with and without glucocorticoids (600 nM). Results are mean ± SEM for $n = 7$ experiments. #, $P < 0.01$ versus corresponding vehicle. **, $P < 0.01$ for differences in angiogenesis between wild-type and 11βHSD1 −/− mice. Angiogenesis was not different between strains in the presence of vehicle or corticosterone but was inhibited by 11-dehydrocorticosterone in wild-type but not 11βHSD1 −/− mice.

steroids were extracted from media by using Sep-Pak C_{18} columns (Waters Millipore). Aortic rings, which contain only 2–3% of the added radioactivity, were not included in the extraction (27). [$^3H_4$]-Corticosterone and [$^3H_4$]-11-dehydrocorticosterone were separated by HPLC and quantified by on-line liquid scintillation counting (16). Enzyme activity was expressed as conversion after subtraction of apparent conversion in negative control wells. Both 11β-reductase (0.65 ± 0.24 pmol/mg) and 11β-dehydrogenase (0.66 ± 0.28 pmol/mg) activities were detected in aortic rings with similar conversion rates as in positive controls: liver for 11βHSD-1 (0.18 ± 0.03 pmol/mg) and kidney for 11βHSD-2 (2.13 ± 1.65 pmol/mg).

To quantify angiogenesis, aortic rings were embedded in 200 μl of steroid-free Matrigel (Becton Dickinson) (Fig. 1) and incubated at 37°C in serum-free MCDB 131, with heparin, ascorbic acid, and GA1000 (Cambrex Biosciences) in the presence and absence of corticosterone (3, 30, 300, and 600 nM), 11-dehydrocorticosterone (300 and 600 nM), the glucocorticoid receptor antagonist RU38486 (10^{-6} M), the mineralocorticoid receptor antagonist spironolactone (10^{-6} M), and/or the nonselective 11βHSD inhibitor carbenoxolone (10^{-6} M). All drugs (Sigma-Aldrich) were dissolved in ethanol and diluted in aqueous solution; final ethanol concentration 1–3% vol/vol. Media were changed every 48 h. Experiments were performed in triplicate. In initial experiments, new vessels were counted daily by using light microscopy (ref. 8 and Fig. 1). From these studies, day 7 was selected as the appropriate time point to examine the effects of glucocorticoids (Fig. 1*B*).

To confirm the nature of apparent new vessels, endothelial cells were identified by uptake of fluorescent-labeled acetylated low-density lipoprotein (Biogenesis, Poole, U.K.) (Fig. 1*A*).

s.c. Sponge Implant Assay. Mice were anesthetized with halothane, and a sterilized sponge cylinder (0.5 cm × 1 cm) (Caligen Foam, Accrington, Lancashire, U.K.) was implanted s.c. on each flank. Sponges contained a silastic insert (Silastic 20 medical grade, Dow Corning) impregnated with vehicle, 2 mg of cortisol or

cortisone, or 5.25 mg of RU38486. Each animal had an intervention-impregnated sponge (steroid or RU38486) on one side and a placebo-impregnated sponge (silastic only) on the other. Such inserts release their impregnated compounds *in vivo* at a constant rate for 3 weeks (28). Human steroids (cortisol and cortisone, equivalent to corticosterone and 11-dehydrocorticosterone) were used to allow distinction from endogenous steroids. In separate experiments (data not shown), angiogenesis in placebo-impregnated sponges was not altered by the presence or absence of a contralateral steroid-treated sponge.

A further cohort of wild-type mice underwent adrenalectomy or sham surgery as described (29) at the time of implantation of untreated sponges. These mice were then maintained on 0.9% saline in place of drinking water.

Twenty days after implantation (10), mice were killed, sponges were excised, and inserts were removed. Sponges were bisected; one half was fixed in 10% formalin and embedded in paraffin wax. Sections (8 μm) were stained with hematoxylin/eosin for identification of blood vessels, as described in ref. 30. The second half of the sponge was weighed, homogenized in 2 ml of sterile PBS at 4°C, and centrifuged (2,000 $\times g$ for 30 min). Steroids were extracted from the supernatant by using ethyl acetate and cortisol quantified by using a specific RIA (Amersham Pharmacia Biotech). Sponge vessel density was determined by using the mean of triplicate Chalkley counts on two sections per sponge (31, 32).

Chronic Coronary Artery Ligation. Wild-type and 11βHSD-1 $-/-$ mice were anesthetized with an i.p. injection of xylazine (0.018 mg/kg), ketamine (100 mg/kg), and atropine (600 mcg/kg) (33). Surgery was performed as described in ref. 34. Briefly, after endotracheal intubation and mechanical ventilation (MiniVent, Harvard Apparatus, Holliston, MA), superficial tissues were dissected, an incision was made in the fourth intercostal space, the pericardium was divided, and the left main descending artery was ligated with 6.0 prolene suture (Ethicon). In sham operated animals, the suture was not ligated. The thoracic wall was closed by layered suturing; the skin was stitched with a continuous suture by using 5-0 Mersilk with a 10-mm 3/8c round-bodied needle (Ethicon). At the time of surgery completion, animals received i.p. atipamazole (5 mg/kg) and s.c. buprenorphine (0.05 mg/kg).

A further cohort of wild-type mice received a s.c. 10 mg implant (28) containing either vehicle or 5.25 mg of RU38486 1 week before coronary artery surgery.

In preliminary experiments, mice were killed on days 1, 3, 5, 7, and 14 after surgery by cervical dislocation. The angiogenic response was well established 7 days after infarction (see Fig. 3*B*), so this interval was selected for comparisons between the groups above. Excised hearts and surgical thoracotomy wounds were fixed in 10% formalin, paraffin embedded, and sectioned at 8 μm. Sections were stained with an anti-von Willebrand factor antibody (DakoCytomation, Cambridgeshire, U.K.) to label endothelial cells and quantify angiogenesis. Hematoxylin and eosin was used to stain sections from hearts collected at day 7 after coronary ligation to measure the area of the left ventricle affected by infarction.

Quantification of vessels within the myocardium was achieved by counting large- and medium-sized vessels as described in ref. 35. Vessels were identified at $\times$400 magnification (Zeiss) in vWF (36–38) stained sections. Counting (39) was performed in the four most vascular fields (two endocardial and two epicardial) by using a 0.0625-mm^2 reticule; the borders of the reticule were within the infarct. The area of left ventricle affected by infarction was determined as a percentage of left ventricular wall area (34) and measured at direct light microscopy; images were captured by using a Research Systems (Imaging Research, St. Catherine's, ON, Canada) photometric camera and analyzed by using in-house scripts.

Wound vessel density was determined in the dermis of vWF-stained sections at $\times$250 light microscopy by using the mean of triplicate Chalkley counts on two sections per wound (31).

Statistics. Data are mean $\pm$ SEM. Comparisons were made by ANOVA with least squares difference post hoc tests. Vessel quantification was performed by investigators "blinded" to the origin of the sections. Interassay and intraassay coefficients of variation in wild-type mice were 17% ($n = 32$) and 22% ($n = 18$), respectively, for vessel number in aortic rings after 7 days in culture; 12% ($n = 6$) and 12% ($n = 6$) for vessel density in sponge implants; 19% ($n = 11$) and 10% ($n = 11$) in day-7 infarcts; and 7% ($n = 4$) and 12% ($n = 4$) for day-7 wounds.

Results

Effects of Glucocorticoids and 11βHSD-1 on Angiogenesis *in Vitro* in Aortic Rings. Both corticosterone and 11-dehydrocorticosterone inhibited angiogenesis in wild-type mouse vessels across a range of physiological concentrations (Fig. 1*B*). The angiostatic effect is mediated by glucocorticoid receptors because it was prevented by the antagonist RU38486 (which has no effect in the absence of steroid) but not by the mineralocorticoid receptor antagonist spironolactone (Fig. 1*C*).

Measurement of relevant product generation confirmed both 11β-reductase (0.65 $\pm$ 0.24 pmol/mg) and 11β-dehydrogenase (0.66 $\pm$ 0.28 pmol/mg) activities in aortic rings with similar conversion rates as in positive controls, liver for 11βHSD-1 (0.18 $\pm$ 0.03 pmol/mg), and kidney for 11βHSD-2 (2.13 $\pm$ 1.65 pmol/mg). Pharmacological inhibition of 11βHSDs in aortic rings was achieved with the nonselective inhibitor carbenoxolone (10^{-6}M), which had no direct effect and prevented the antiangiogenic effect of 11-dehydrocorticosterone but not corticosterone (Fig. 1*Di*).

To confirm the role of 11βHSD-1, aortic rings were obtained from homozygous 11βHSD-1 null ($-/-$) mice congenic on a C57Bl6J genetic background (26) and C57Bl6J controls. Angiogenesis in aortic rings from 11βHSD-1 $-/-$ mice was similar to that in wild-type controls in the absence of steroid and inhibited to a similar degree by corticosterone. However, 11-dehydrocorticosterone did not inhibit angiogenesis in vessels from 11βHSD-1 $-/-$ mice (Fig. 1*Dii*).

Effect of Endogenous Glucocorticoids and 11βHSD-1 on Angiogenesis in s.c. Sponge Implants *in Vivo*. Placebo-impregnated sponges excised after 20 days (10) were red on gross inspection with a lace-like covering of blood vessels. At histology, there was an inflammatory infiltrate and an abundance of blood vessels (Fig. 2*Ai*). Sponges from adrenalectomized animals and sponges impregnated with the glucocorticoid receptor antagonist RU38486 both exhibited enhanced angiogenesis (Fig. 2*Bi*) in wild-type mice.

To test the effects of 11-hydroxy and 11-keto-glucocorticoids we used the "human" steroids cortisol and cortisone, which allowed measurement of steroid concentrations within the sponge independently of endogenous murine corticosterone and 11-dehydrocorticosterone (Table 1). In wild-type C57Bl6J mice, both cortisol and cortisone inhibited angiogenesis *in vivo* (Fig. 2 *Aii* and *Bii*). In 11βHSD-1 null mice, angiogenesis was increased in placebo-impregnated sponges. Impregnation with cortisol produced similar cortisol concentrations in wild-type and 11βHSD-1 null mice (Table 1) and inhibited angiogenesis to a similar degree (Fig. 2*Bii*). However, impregnation with cortisone, in contrast with its effects in wild-type controls, did not elevate sponge cortisol concentrations in 11βHSD-1 null mice (Table 1) and did not inhibit angiogenesis (Fig. 2*Bii*).

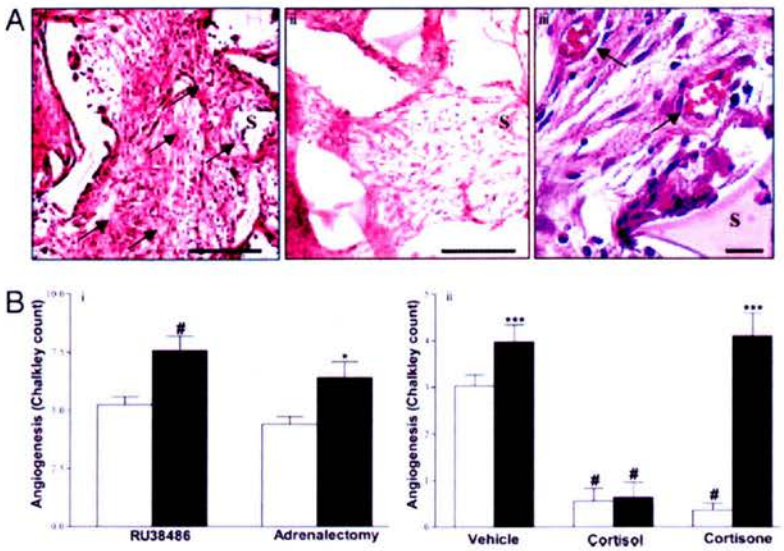

Fig. 2. Angiogenesis in s.c. implanted sponges. (*A*) Light microscopy of hematoxylin/eosin stained sponge 8-μm sections from wild-type mice: vehicle (*i*) and cortisol-treated (*ii*) sponge (Scale bar: 400 μm) and vehicle-treated sponge at high power (*iii*) (Scale bar: 50 μm.) Sponges were covered with a fibroblast-rich fibrous coat and were infiltrated with inflammatory neutrophils and lymphocytes. Placebo-treated sponges alone were also infiltrated with an organized matrix and an abundance of blood vessels (black arrows). S denotes sponge matrix. (*Bi*) Sponges from C57Bl6J wild-type ($n = 6$) mice. Exposure to RU38486 or adrenalectomy (filled bars) compared with placebo or sham surgery (open bars). Results are mean ± SEM. #, $P < 0.01$ versus vehicle; *, $P < 0.02$ versus sham. New vessel formation was greater in RU38486-impregnated sponges or sponges from adrenalectomized mice versus their relevant controls. (*Bii*) Sponges from C57Bl6J wild-type (open bars, $n = 12$) or 11βHSD1 $-/-$ (filled bars, $n = 6$) mice with and without glucocorticoids. Results are mean ± SEM. #, $P < 0.001$ versus corresponding vehicle. ***, $P < 0.001$ for differences between wild type and 11βHSD1 $-/-$. Placebo-impregnated sponges exhibited an increased angiogenic response in 11βHSD1 $-/-$ compared to wild-type mice. Cortisol inhibited angiogenesis in both strains, but cortisone inhibited angiogenesis only in wild-type mice.

Effect of Endogenous Glucocorticoids and 11βHSD-1 on Myocardial Revascularization After Coronary Artery Ligation. At day 7, the proportional area of the left ventricular myocardium affected by coronary artery ligation was similar in all treatment groups (41.8 ± 6.2% in vehicle versus 45.5 ± 4.8% in RU38486 and 44.2 ± 3.4% in wild types versus 44.2 ± 2.6% in 11βHSD-1$^{-/-}$). RU38486 increased angiogenesis in the left ventricle after infarction in wild-type mice (Fig. 3*Bii*).

There was no difference in myocardial vascularity between sham-operated wild-type and 11βHSD-1 null mice. In contrast, 7 days after coronary artery ligation, 11βHSD-1 null mice exhibited enhanced revascularization in the infarcted myocardium (Fig. 3*Bii*),

Effect of Endogenous Glucocorticoids and 11βHSD-1 on Angiogenesis in Cutaneous Surgical Wounds. New vessel formation was examined in cutaneous surgical wounds in mice that underwent thoracotomy for the coronary artery ligation studies (Fig. 3*c*). The dermal angiogenic response was greater in RU38486-treated mice (4.8 ± 0.29 Chalkley count versus vehicle 3.5 ± 0.21; $P < 0.01$) and in 11βHSD-1 null mice (5.1 ± 0.27 Chalkley count; $P < 0.01$) in comparison to wild-type controls (3.5 ± 0.25 Chalkley count).

Discussion

Folkman *et al.* described the angiostatic effects of pharmacological glucocorticoids *in vitro* >20 years ago (11), and these effects have been confirmed *in vivo* (3, 10). Here, we show that the angiostatic effect occurs at physiological concentrations of glucocorticoids and is mediated by glucocorticoid receptors, and that endogenous glucocorticoids tonically repress angiogenic responses. Moreover, we show that 11βHSD1, by regenerating active glucocorticoids locally, amplifies the angiostatic effect of glucocorticoids and, thereby, constrains the angiogenic response after ischemia and injury.

These observations raise the intriguing possibility that local variations in cortisol levels or in tissue sensitivity to cortisol are key determinants of angiogenesis in disease. It is well recognized that, in Cushing's syndrome, glucocorticoid excess is associated with impaired wound healing (40). More recently, we showed that exogenous glucocorticoid therapy is associated not only with increased incidence of myocardial infarction but also with an unexpected increase in prevalence of heart failure (41, 42), suggesting an impact on the outcome and the incidence of cardiovascular disease. More subtle variations in cortisol secretion and action, including variations in responses to stress, have been described in many populations and have been related to risk factors for occlusive vascular disease, mood, development in early life, gender, age, etc. (43–45). We now suggest that effects of cortisol on angiogenesis could explain the links between these quantitative traits in the population and the health outcomes from vascular disease and, perhaps, from other diseases involving angiogenesis, including neoplasia. If so, then therapies, which reduce glucocorticoid action within ischemic tissue, may be valuable in improving collateral perfusion. This result cannot be achieved safely with systemic antiglucocorticoid therapy that is likely to lead to Addisonian crisis after a severe stressor such as myocardial infarction. The role of 11βHSD-1 described here offers an opportunity for tissue-specific targeting of therapy.

We described the presence of 11βHSD-1 in the vessel wall >10 years ago (16), but its importance has remained obscure. The observations that nonselective 11βHSD inhibitors influence vascular tone (27, 46) can be attributed to effects on the 11βHSD-2 isozyme that catalyses inactivation of glucocorticoids within endothelial cells (15, 22). Here, we show that regeneration of glucocorticoids by 11βHSD-1 in isolated aortae amplifies their angiostatic effect. We found no evidence that dehydrogenase 11βHSD-2 influences angiogenesis *in vitro* because the nonselective 11βHSD inhibitor carbenoxolone did not potentiate the angiostatic effect of corticosterone.

In vivo 11βHSD-1 null mice have no obvious difference in vascular structure in healthy tissues. Normal vascular development occurs in other models of altered angiogenesis in

Table 1. Cortisol concentration in sponge homogenates from wild-type and 11βHSD1 $-/-$ homozygous null mice

Strain	Steroid impregnated	Cortisol level (ng/g sponge)	
		Ipsilateral steroid-treated sponge	Contralateral placebo-treated sponge
Wild type	Cortisol	4,271 ± 186#	161 ± 18
	Cortisone	295 ± 25#**	98 ± 19
11βHSD-1 $-/-$	Cortisol	3,775 ± 1,703#	135 ± 46
	Cortisone	87 ± 11	90 ± 30

Results are mean ± SEM for $n = 3$–6 experiments. #, $P < 0.01$ versus contralateral placebo. **, $P < 0.01$ for comparison of wild type and 11βHSD1 $-/-$.

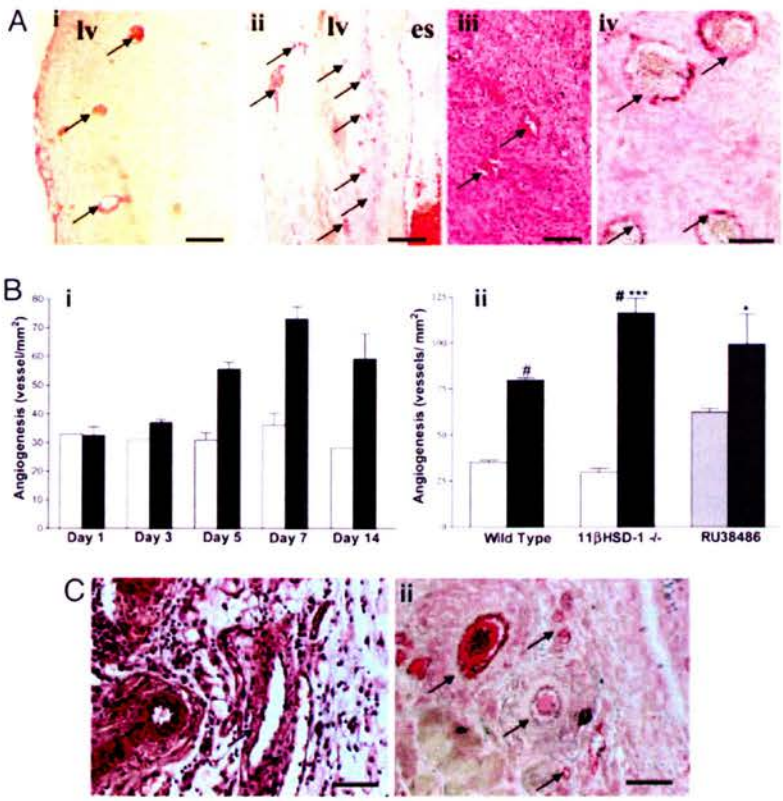

Fig. 3. Effect of injury on angiogenesis in mouse myocardium and skin. (*A*) Light microscopy (×50) of anti-von Willebrand factor immunostaining with fast red chromogen substrate in day 7 wild-type sham (*i*) and infarcted (*ii*) hearts. Scattered medium and large vessels were detected in sham hearts. In contrast, many more vessels were observed in the healing myocardium after infarction. Black arrows, vessels; lv, left ventricle, es, endocardial surface. (Scale bar: 100 μm.) (*iii*) Medium power (×100) light microscopy of hematoxylin/eosin staining in day-7 wild-type infarcted heart. (Scale bar: 100 μm.) (*iv*) High power (×400) light microscopy of anti-von Willebrand immunostaining in day-7 wild-type infarcted heart; (Scale bar: 100 μm.) The vascularity of the infarcted myocardium was increased and multiple vessels containing erythrocytes were observed (black arrows indicate vessels). (*Bi*) Vascularity of myocardium of wild-type mouse hearts after ligation (filled bars, n = 3–11) or sham surgery (open bars, n = 1–6). Sham-operated animals show a constant vascularity in contrast to CCL animals in which vessel counts increase with time, achieving a maximum at day 7. (*Bii*) Day-7 hearts from wild-type and 11βHSD1 $-/-$ mice. Ligation (filled bars) in wild-type and 11βHSD1 $-/-$ increased angiogenesis in comparison to sham (open bars) (wild type, n = 6 sham and n = 11 ligations; 11βHSD1$-/-$, n = 5 sham and n = 10 ligations). Ligations in mice that received RU38486 (dark gray bars, n = 6) induced greater myocardial angiogenesis in comparison to vehicle-treated ligated controls (light gray bars, n = 3). Results are mean ± SEM. #, $P < 0.001$ versus corresponding sham. ***, $P < 0.001$ for differences between wild-type and 11HSD1 $-/-$.*, $P < 0.02$ for differences between coronary artery ligated wild-type mice treated with RU38486 or vehicle. (*C*) Identification of blood vessels in 7-day-old cutaneous wounds from wild-type mice stained with hematoxylin/eosin (*i*) or an antibody against von Willebrand factor (*ii*). (Magnification: ×400; scale bars: 100 μm.)

which the abnormality is apparent only in adult pathology (47), thus reflecting the distinct pathways underlying vasculogenesis and adult angiogenesis. However, when angiogenesis is stimulated in adult mice, we found that 11βHSD-1 amplifies the angiostatic effect of endogenous glucocorticoids. In s.c. sponge implants, this effect is local, rather than systemic, because angiogenesis in contralateral sponges was unaffected. Moreover, cortisol concentrations in the sponges were lower after impregnation with cortisone than with cortisol, suggesting that it is the generation of cortisol locally within the cells that express 11β-HSD1, rather than levels of cortisol in the interstitial fluid of the sponge, which determines the angiostatic effect. Finally, the relevance of 11βHSD-1 was confirmed by the demonstration that 11βHSD-1 null mice exhibit greater angiogenic responses in wounds and infarcted myocardium. In these studies, immunohistochemical localization of vWF enabled quantification of large- and medium-sized vessels (35) but not the entire population of endothelial cells in a section. Thus, all of the vessels included in the quantification are likely to be functional.

It is possible that these observations reflect 11βHSD-1 activity either within the vessel wall or in the inflammatory infiltrate that accompanies angiogenesis in all of these *in vivo* models. 11βHSD-1 is expressed in macrophages (48), and regeneration of glucocorticoids enhances phagocytosis of apoptotic neutrophils (49), hence absence of 11βHSD-1 may confer a prolonged and enhanced acute inflammatory response that, in turn, might stimulate angiogenesis. However, 11βHSD-1 in the inflammatory infiltrate cannot explain the influence of 11βHSD-1 in isolated aortic rings. The findings in the isolated aortic ring model confirm that vessel wall 11βHSD-1 moderates the angiostatic influence of glucocorticoids and confirms that regeneration of active glucocorticoids within vascular smooth muscle cell can inhibit angiogenic processes. Although the *in vivo* models validated the isolated aortic ring findings, it is apparent nonetheless that inflammatory cytokines induce 11βHSD-1 expression in a variety of cell types (13), including in vascular smooth muscle cells (23), so that the contribution of 11βHSD-1 within the vessel wall may be intimately related with the extent of the inflammatory response.

Angiogenesis is crucially dependent on endothelial cells producing key factors such as vascular endothelial growth factor (VEGF) and forming a *de novo* collagen basement membrane to allow structured cell proliferation (24). In the chick chorioallantoic membrane, glucocorticoids alter endothelial cell morphology and collagen production (7, 9). It has also been proposed that glucocorticoid effects are mediated by inhibition of endothelial VEGF transcription and endothelial nitric oxide production (19, 50). However, in keeping with a role for 11βHSD-1, the effect of glucocorticoids may be mediated within vascular smooth muscle, where inhibition of matrix metalloproteinase production (51) may alter the efficacy of endothelium-dependent new vessel formation, and antiproliferative effects (20) may attenuate formation of vessel walls around endothelial cell buds.

In contrast to the established effects of supraphysiological concentrations of glucocorticoids, the influence of endogenous glucocorticoids on angiogenesis has until now remained unclear. The current findings *in vitro* and *in vivo* confirm a physiological role for endogenous glucocorticoids to regulate angiogenesis and highlight the significance of vascular 11βHSD-1 in modulating this effect. The wider relevance of these findings to pathology is illustrated in the models of wound healing and myocardial infarction. These findings may lead to therapeutic approaches to enhance angiogenesis by preventing glucocorticoid action. Although systemic glucocorticoid receptor blockade is unlikely to be successful as a

treatment in the short term (because of adverse effects of preventing the normal cortisol-dependent stress response) or in the long term (because of compensatory activation of the hypothalamic pituitary adrenal axis), the current data suggest that manipulation of local 11βHSD-1 offers a more targeted approach to the blood vessel wall. 11βHSD-1 inhibitors are already being developed for reducing risk factors for cardiovascular disease (45), including in type 2 diabetes mellitus and obesity. These results suggest that pharmacological inhibition of 11βHSD-1 may also be valuable in ischemic heart disease and impaired wound healing.

We thank Profs. John Mullins and Jonathan Seckl (University of Edinburgh) for their guidance and kind provision of 11βHSD-1 $-/-$ mice and Jill Harrison, Dr. Janice Paterson, Dr. Paul Perry, Dr. Roy Bicknell, Sandra Peake, and Dr. Kairbaan Hodivala-Dilke for assistance and advice. This work was supported by the Wellcome Trust, British Heart Foundation, and Medical Research Council.

1. Kurotobi, T., Sato, H., Kinjo, K., Nakatani, D., Mizuno, H., Shimizu, M., Imai, K., Hirayama, A., Kodama, K. & Hori, M. (2004) *J. Am. Coll. Cardiol.* **44,** 28–34.
2. Vernieri, F., Pasqualetti, P., Matteis, M., Passarelli, F., Troisi, E., Rossini, P. M., Caltagirone, C. & Silvestrini, M. (2001) *Stroke* **32,** 1552–1558.
3. Hasan, Q., Tan, S. T., Gush, J., Peters, S. G. & Davis, P. F. (2000) *Pediatrics* **105,** 117–120.
4. Aiello, L. P. & Wong, J. S. (2000) *Kidney Int. Suppl.* **77,** S113–S119.
5. Siemann, D. W., Chaplin, D. J. & Horsman, M. R. (2004) *Cancer* **100,** 2491–2499.
6. Kinnaird, T., Stabile, E., Epstein, S. E. & Fuchs, S. (2003) *J. Interv. Cardiol.* **16,** 289–297.
7. Maragoudakis, M. E., Sarmonika, M. & Panoutsacopoulou, M. (1989) *J. Pharmacol. Exp. Ther.* **251,** 679–682.
8. Nicosia, R. F. & Ottinetti, A. (1990) *Lab. Invest.* **63,** 115–122.
9. Folkman, J. & Ingber, D. E. (1987) *Ann. Surg.* **206,** 374–383.
10. Hori, Y., Hu, D. E., Yasui, K., Smither, R. L., Gresham, G. A. & Fan, T. P. (1996) *Br. J. Pharmacol.* **118,** 1584–1591.
11. Folkman, J., Langer, R., Linhardt, R. J., Haudenschild, C. & Taylor, S. (1983) *Science* **221,** 719–725.
12. Stewart, P. M. & Krozowski, Z. S. (1999) *Vitam. Horm.* **57,** 249–324.
13. Seckl, J. R. & Walker, B. R. (2001) *Endocrinology* **142,** 1371–1376.
14. Smith, R. E., Little, P. J., Maguire, J. A., Stein-Oakley, A. N. & Krozowski, Z. S. (1996) *Clin. Exp. Pharmacol. Physiol.* **23,** 549–551.
15. Christy, C., Hadoke, P. W. F., Paterson, J. M., Mullins, J. J., Seckl, J. R. & Walker, B. R. (2003) *Hypertension* **42,** 580–587.
16. Walker, B. R., Yau, J. L., Brett, L. P., Seckl, J. R., Monder, C., Williams, B. C. & Edwards, C. R. W. (1991) *Endocrinology* **129,** 3305–3312.
17. Takeda, Y., Miyamori, I., Yoneda, T., Ito, Y. & Takeda, R. (1994) *Life Sci.* **54,** 281–285.
18. Brem, A. S., Bina, R. B., King, T. & Morris, D. J. (1995) *Steroids* **60,** 406–410.
19. Ullian, M. E. (1999) *Cardiovasc. Res.* **41,** 55–64.
20. Longenecker, J. P., Kilty, L. A. & Johnson, L. K. (1984) *J. Cell Biol.* **98,** 534–540.
21. Versaci, F., Gaspardone, A., Tomai, F., Ribichini, F., Russo, P., Proietti, I., Ghini, A. S., Ferrero, V., Chiariello, L., Gioffre, P. A., *et al.* (2002) *J. Am. Coll. Cardiol.* **40,** 1935–1942.
22. Hadoke, P. W. F., Christy, C., Kotelevtsev, Y. V., Williams, B. C., Kenyon, C. J., Seckl, J. R., Mullins, J. J. & Walker, B. R. (2001) *Circulation* **104,** 2832–2837.
23. Cai, T. Q., Wong, B. M., Mundt, S. S., Thieringer, R., Wright, S. D. & Hermanowski-Vosatka, A. (2001) *J. Steroid Biochem.* **77,** 117–122.
24. Conway, E. M., Collen, D. & Carmeliet, P. (2001) *Cardiovasc. Res.* **49,** 507–521.
25. Kotelevtsev, Y. V., Holmes, M. C., Burchell, A., Houston, P. M., Scholl, D., Jamieson, P., Best, R., Brown, R. W., Edwards, C. R. W., Seckl, J. R. & Mullins, J. J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 14924–14929.
26. Morton, N. M., Paterson, J. M., Masuzaki, H., Holmes, M. C., Staels, B., Fievet, C., Walker, B. R., Flier, J. S., Mullins, J. J. & Seckl, J. R. (2004) *Diabetes* **53,** 931–938.
27. Souness, G. W., Brem, A. S. & Morris, D. J. (2002) *Steroids* **67,** 195–201.
28. Soro, A., Panarelli, M., Holloway, C. D., Fraser, R. & Kenyon, C. J. (1997) *Steroids* **62,** 388–394.
29. Livingstone, D. E. W., Kenyon, C. J. & Walker, B. R. (2000) *J. Endocrinol.* **167,** 533–539.
30. Andrade, S. P., Fan, T. P. & Lewis, G. P. (1987) *Br. J. Exp. Pathol.* **68,** 755–766.
31. Fox, S. B., Leek, R. D., Weekes, M. P., Whitehouse, R. M., Gatter, K. C. & Harris, A. L. (1995) *J. Pathol.* **177,** 275–283.
32. Hague, S., MacKenzie, I. Z., Bicknell, R. & Rees, M. C. (2002) *Hum. Reprod.* **17,** 786–793.
33. Mora, A., Davies, A. M., Bertrand, L., Sharif, I., Budas, G. R., Jovanovic, S., Mouton, V., Kahn, C. R., Lucocq, J. M., Gray, G. A., *et al.* (2003) *EMBO J.* **22,** 4666–4676.
34. Lutgens, E., Daemen, M. J., de Muinck, E. D., Debets, J., Leenders, P. & Smits, J. F. (1999) *Cardiovasc. Res.* **41,** 586–593.
35. Heymans, S., Luttun, A., Nuyens, D., Theilmeier, G., Creemers, E., Moons, L., Dyspersin, G. D., Cleutjens, J. P., Shipley, M., Angellilo, A., *et al.* (1999) *Nat. Med.* **5,** 1135–1142.
36. Jayasankar, V., Woo, Y. J., Bish, L. T., Pirolli, T. J., Chatterjee, S., Berry, M. F., Burdick, J., Gardner, T. J. & Sweeney, H. L. (2003) *Circulation* **108,** 230–236.
37. Schwarz, E. R., Meven, D. A., Sulemanjee, N. Z., Kersting, P. H., Tussing, T., Skobel, E. C., Hanrath, P. & Uretsky, B. F. (2004) *J. Cardiovasc. Pharmacol. Ther.* **9,** 279–289.
38. Li, W., Tanaka, K., Ihaya, A., Fujibayashi, Y., Takamatsu, S., Morioka, K., Sasaki, M., Uesaka, T., Kimura, T., Yamada, N., *et al.* (2005) *Am. J. Physiol.* **288,** H408–H415.
39. Virag, J. I. & Murry, C. E. (2003) *Am. J. Pathol.* **163,** 2433–2440.
40. Gordon, C. B., Li, D. G., Stagg, C. A., Manson, P. & Udelsman, R. (1994) *Surgery* **116,** 1082–1087.
41. Souverain, P. C., Berard, A., van Staa, T. P., Cooper, C., Leufkens, H. G. M. & Walker, B. R. (2004) *Heart* **90,** 859–865.
42. Wei, L., MacDonald, T. M. & Walker, B. R. (2004) *Ann. Intern. Med.* **141,** 764–770.
43. Phillips, D. I. W., Barker, D. J. P., Fall, C. H. D., Whorwood, C. B., Seckl, J. R., Wood, P. J. & Walker, B. R. (1998) *J. Clin. Endocrinol. Metab.* **83,** 757–760.
44. McEwen, B. S. (1999) *Front. Neuroendocrinol.* **20,** 49–70.
45. Walker, B. R. & Seckl, J. R. (2004) *Expert Opin. Ther. Targets* **7,** 771–783.
46. Teelucksingh, S., Mackie, A. D. R., Burt, D., McIntyre, M. A., Brett, L. & Edwards, C. R. W. (1990) *Lancet* **335,** 1060–1063.
47. Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y., Bono, F., Devy, L., Beck, H., *et al.* (2001) *Nat. Med.* **7,** 575–583.
48. Thieringer, R., Le Grand, C. B., Carbin, L., Cai, T.-Q., Wong, B. & Wright, S. D. (2001) *J. Immunol.* **167,** 30–35.
49. Rossi, A., Liu, Y., Cousin, J. M., Dransfield, I., Seckl, J. R., Haslett, C. & Savill, J. (1999) *J. Immunol.* **162,** 3639–3646.
50. Nauck, M., Karakiulakis, G., Perruchoud, A. P., Papakonstantinou, E. & Roth, M. (1998) *Eur. J. Pharmacol.* **341,** 309–315.
51. Pross, C., Farooq, M. M., Lane, J. S., Angle, N., Tomono, C. K., Xavier, A. E., Freischlag, J. A., Collins, A. E., Law, R. E. & Gelabert, H. A. (2002) *J. Vasc. Surg.* **35,** 1253–1259.

Appendix

E-Mail to the Editor of PNAS

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